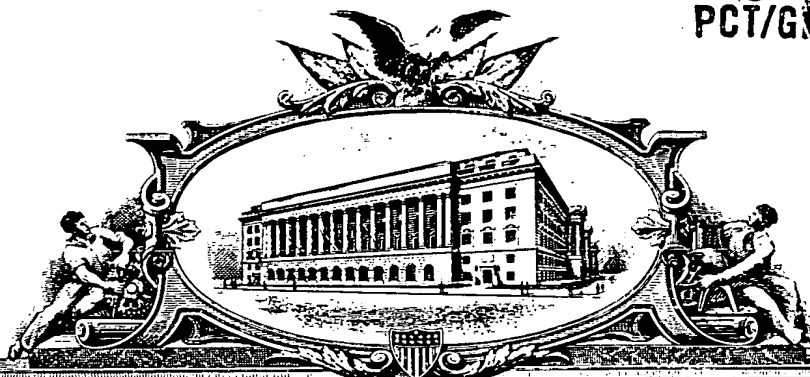


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APPLICATION NUMBER: 60/024,188

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## PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2)

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## TITLE OF THE INVENTION (200 characters max)

POLYKETIDES AND THEIR SYNTHESIS

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## ENCLOSED APPLICATION PARTS (check all that apply)

<input checked="" type="checkbox"/>	Specification	Number of Pages	98	<input type="checkbox"/>	Small Entity Statement
<input checked="" type="checkbox"/>	Drawings	Number of Sheets	19	<input checked="" type="checkbox"/>	Other (specify):
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Yes, the name of the U.S. Government Agency and the Government contract number are:

Respectfully submitted,

SIGNATURE:

TYPED or PRINTED NAME Walter H. Dreger

Date

19 August 1996

REGISTRATION NO.  
(if appropriate)

24,190

Additional inventors are being named on separately numbered sheets attached hereto.

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POLYKETIDES AND THEIR SYNTHESIS

The present invention relates to novel polyketides and methods and means for preparing them by recombinant synthesis. Polyketide biosynthetic genes or portions of them, which may be derived from different polyketide biosynthetic gene clusters are manipulated to allow the production of specific novel hybrid polyketides of predicted structure. The invention also relates to novel host-vector systems allowing increased levels of production of both natural and non-natural polyketides.

Polyketides are a large and structurally diverse class of natural products that includes many compounds possessing antibiotic or other pharmacological properties, such as erythromycin, tetracyclines, rapamycin, avermectin and FK506. In particular, polyketides are abundantly produced by Streptomyces and related actinomycete bacteria. They are synthesised by the repeated stepwise condensation of acylthioesters in a manner analogous to that of fatty acid biosynthesis. The greater structural diversity found among natural polyketides arises from the selection of (usually) acetate or propionate as "starter" or "extender" units; and from the differing degree of processing of the  $\beta$ -keto group observed after each condensation. Examples of processing steps include reduction to  $\beta$ -hydroxyacyl-, reduction followed by dehydration to 2-enoyl-, and complete reduction to the saturated acylthioester. The stereochemical outcome of these processing steps is also specified for each cycle of

chain extension.

The biosynthesis of polyketides is initiated by a group of chain-forming enzymes known as polyketide synthases. Two classes of polyketide synthase (PKS) have been described in actinomycetes. One class, named Type I PKSs, represented by the PKSs for the macrolides erythromycin, avermectin and rapamycin (Figure 1), consists of a different set or "module" of enzymes for each cycle of polyketide chain extension (Figure 2) (Cortes, J. et al. Nature (1990) 348:176-178; Donadio, S. et al. Science (1991) 252:675-679; MacNeil, D. J. et al. Gene (1992), 115:119-125; Schwecke, T. et al. Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843). Note: the term "natural module" as used herein refers to the set of contiguous domains, from a  $\beta$ -ketoacylsynthase ("KS") gene to the next acyl carrier protein ("ACP") gene, which accomplishes one cycle of polyketide chain extension. The term "combinatorial module" is used to refer to any group of contiguous domains (and domain parts), extending from a first point in a first natural module, to a second equivalent point in a second natural module. The first and second points will generally be in core domains which are present in all modules, ie both at equivalent points of respective KS, AT (acyl transferase) or ACP domains. The length of polyketide formed has been altered, in the case of erythromycin biosynthesis, by specific relocation using genetic engineering of the enzymatic domain of the

erythromycin-producing PKS that contains the chain-releasing thioesterase/cyclase activity (Cortes, J. et al. Science (1995) 268:1487-1489; Kao, C. M. et al. J. Am. Chem. Soc. (1995) 117:9105-9106)

5

In-frame deletion of the DNA encoding part of the ketoreductase domain in module 5 of the erythromycin-producing PKS, (also known as 6-deoxyerythronolide B synthase, DEBS) has been shown to lead to the formation of erythromycin analogues 5,6-dideoxy-3- $\alpha$ -mycarosyl-5-oxoerythronolide B, 5,6-dideoxy-5-oxoerythronolide B and 5,6-dideoxy-6,6 $\alpha$ -epoxy-5-oxoerythronolide B (Donadio, S. et al. Science, (1991) 252:675-679). Likewise, alteration of active site residues in the enoylreductase domain of module 4 in DEBS, by genetic engineering of the corresponding PKS-encoding DNA and its introduction into *Saccharopolyspora erythraea*, led to the production of  $\Delta$ 6,7-anhydroerythromycin C (Donadio S. et al. Proc. Natl. Acad. Sci. USA (1993) 90:7119-7123).

International Patent Application number WO 93/13663 describes additional types of genetic manipulation of the DEBS genes that are capable of producing altered polyketides. However, many such attempts are reported to have been unproductive (Hutchinson C. R. and Fujii, I. Annu. Rev. Microbiol. (1995) 49:201-238, at p.231), and no further examples of altered polyketides have been reported. The complete DNA sequence of the genes from

Streptomyces hygroscopicus that encode the modular Type I PKS governing the biosynthesis of the macrocyclic immunosuppressant polyketide rapamycin has been disclosed (Schwecke, T. et al. (1995) Proc. Natl. Acad. Sci. USA 92:7839-7843) (Figure 3). The DNA sequence is deposited in the EMBL/Genbank Database under the accession number X86780.

The second class of PKS, named Type II PKSs, is represented by the synthases for aromatic compounds. Type II PKSs contain only a single set of enzymatic activities for chain extension and these are re-used as appropriate in successive cycles (Bibb, M. J. et al. EMBO J. (1989) 8:2727-2736; Sherman, D. H. et al. EMBO J. (1989) 8:2717-2725; Fernandez-Moreno, M. A. et al. J. Biol. Chem. (1992) 267:19278-19290). The "extender" units for the Type II PKSs are usually acetate units, and the presence of specific cyclases dictates the preferred pathway for cyclisation of the completed chain into an aromatic product (Hutchinson, C. R. and Fujii, I. Annu. Rev. Microbiol. (1995) 49:201-238). Hybrid polyketides have been obtained by the introduction of cloned Type II PKS gene-containing DNA into another strain containing a different Type II PKS gene cluster, for example by introduction of DNA derived from the gene cluster for actinorhodin, a blue-pigmented polyketide from Streptomyces coelicolor, into an anthraquinone polyketide-producing strain of Streptomyces galileus (Bartel, P. L. et al. J. Bacteriol. (1990) 172:4816-4826).



International Patent Application Number WO 95/08548 describes the replacement of actinorhodin PKS genes by heterologous DNA from other Type II PKS clusters, to obtain hybrid polyketides. The same International Patent Application WO 95/08548 describes the construction of a strain of *Streptomyces coelicolor* which substantially lacks the native gene cluster for actinorhodin, and the use in that strain of a plasmid vector pRM5 derived from the low-copy number plasmid vector SCP2\* isolated from *Streptomyces coelicolor* (Bibb, M. J. and Hopwood, D. A. J. Gen. Microbiol. (1981) 126:427) and in which heterologous PKS-containing DNA may be expressed under the control of the divergent act I/act III promoter region of the actinorhodin gene cluster (Fernandez-Moreno, M. A. et al. J. Biol. Chem. (1992) 267:19278-19290). The plasmid pRM5 also contains DNA from the actinorhodin biosynthetic gene cluster encoding the gene for a specific activator protein, Act II-orf4. The Act II-orf4 protein is required for transcription of the genes placed under the control of the act I/act III bidirectional promoter and activates expression during the transition from growth to stationary phase in the vegetative mycelium (Hallam, S. E. et al. Gene (1988) 74:305-320).

Type II PKS clusters in *Streptomyces* are known to be activated by pathway-specific activator genes (Narva, K.E. and Feitelson, J. S. J. Bacteriol. (1990) 172:326-333; Stutzman-Engwall, K. J. et al. J. Bacteriol (1992) 174:144-154; Fernandez-Moreno, M. et al. Cell (1991)

66:769-780; Takano, E. et al. Mol. Microbiol. (1992)  
7:837-845; Takano, E. et al. Mol. Microbiol. (1992)  
6:2797-2804) whose gene product is required for  
transcription from specific promoters. The gene product  
5 of the activator genes is speculated to act by binding to  
specific DNA sequences in promoters of the PKS gene  
cluster in which the activator gene is located  
(Stutzman-Engwall, K. J. et al. J. Bacteriol (1992)  
174:144-154; Takano, E. et al. Mol. Microbiol. (1992)  
10 7:837-845). The DnrI gene product complements a mutation  
in the actII-orf4 gene of *S. coelicolor*, implying that  
DnrI and ActII-orf4 proteins act on similar targets. A  
gene (*srmR*) has been described (EP 0 524 832 A2) that is  
located near the Type I PKS gene cluster for the macrolide  
15 polyketide spiramycin, this gene specifically activates  
the production of the macrolide polyketide spiramycin, but  
no other examples have been found of such a gene. Also,  
no homologues of the ActII-orf4/DnrI/RedD family of  
activators have been described that act on Type I PKS  
20 genes.

Although large numbers of therapeutically important  
polyketides have been identified, there remains a need to  
obtain novel polyketides that have enhanced properties or  
possess completely novel bioactivity. The complex  
25 polyketides produced by modular Type I PKSs are  
particularly valuable, in that they include compounds with  
known utility as antihelminthics, insecticides,  
immunosuppressants, antifungal or antibacterial agents.

Because of their structural complexity, such novel polyketides are not readily obtainable by total chemical synthesis, or by chemical modifications of known polyketides.

5        There is a need to develop reliable and specific ways of deploying individual modules in practice so that all, or a large fraction, of hybrid PKS genes that are constructed, are viable and produce the desired polyketide product. This is particularly true if it is desired to  
10 create large numbers of individual PKS gene sets using Type I modular PKS genes in a combinatorial fashion, where it will not be feasible to analyse all members of the set. Such libraries of polyketides offer a highly attractive alternative to the random screening of soil samples for  
15 the discovery of novel polyketides with valuable bioactive properties.

Similarly, although specific host-vector combinations have been reported that allow the controlled expression of heterologous genes in certain *Streptomyces* as for example  
20 using induction by added thiostrepton as described for *Streptomyces lividans* 66 and *Streptomyces coelicolor* (Takano, E. et al. *Gene* (1995) 166:133-137) and by utilising nutritional signals at the onset of differentiation, as for *Streptomyces coelicolor* in  
25 International Patent Application number WO 95/08548, there remains an important need for the development of general methods of controlling and even enhancing the expression of a structural gene, or of a set of structural genes,

that governs the biosynthesis of a potentially valuable secondary metabolite such as one of the complex polyketides, in an engineered strain of *Streptomyces* or of a related filamentous bacterium.

5 One aspect of the invention arises from our appreciation that a PKS gene assembly (particularly of type I) encodes a loading module which is followed by extension modules. Thus Fig. 2 shows the organisation of the DEBS genes. The first open reading frame encodes the  
10 first multi-enzyme or cassette (DEBS1) which consists of three modules: the loading module (ery-load) and two extension modules (modules 1 and 2). The loading module comprises an acyl transferase and an acyl carrier protein. This may be contrasted with Fig. 1 of WO93/13663 (referred  
15 to above). This shows ORF1 to consist of only two modules, the first of which is in fact both the loading module and the first extension module.

In one aspect the invention concerns the production of a hybrid PKS gene assembly comprising a loading module  
20 and at least one, and preferably a plurality, of extension modules by assembling together a first nucleic acid portion or portions encoding at least one domain of a first type I PKS with a second nucleic acid portion or portions encoding at least one type I PKS domain which is  
25 heterologous to said first PKS. Generally the nucleic acids are DNA. The first and second portions may each encode domain(s) of respective different PKS's.

Preferably the hybrid PKS encodes a loading module

and from 1 to 6 extension modules within any give cassette. More preferably there are at least 2 extension modules. NB: products resulting from many more than 6 modules can result from assemblies of synthases (c.f. 5 rapamycin).

The first portion may encode a loading module, while the second portion encodes one or more extension modules. Alternatively the first portion(s) may encode all or part of a loading module, the first two extension modules, and 10 a chain terminating enzyme (generally a thioesterase), e.g. of erythromycin PKS, and the second portion(s) correspond to one or more domains and/or modules of a different PKS.

It is particularly useful to provide a hybrid PKS 15 gene assembly in which the loading module is heterologous to the extension modules and is such as to lead to a polyketide having an altered starter unit. NB: This is a concept quite unknown to the prior art since this does not recognise the existence of loading modules. WO93/13663 20 refers to altering PKS genes by inactivating a single function (i.e. a single enzyme) or affecting "an entire module" by deletion, insertion or replacement thereof. But in their terms the loading assembly is not a module.

If the loading module is one which accepts many 25 different carboxylic acid units then the hybrid gene assembly can be used to produce many different polyketides. For example a hybrid gene assembly may employ nucleic acid encoding an avr loading module with

ery extender modules. A loading module may accept unnatural acid units. Alternatively or additionally we may alter the end of a gene assembly. Thus the normal chain terminating enzyme of a PKS (usually thioesterase) may be replaced by an enzyme leading to a different type of product. Thus use may be made of the enzyme from the rapamycin system that connects the polyketide chain to an aminoacid chain. This can be used to synthesise polypeptide/polyketide combinations, e.g. for producing  $\beta$ -lactam derivatives.

Of course one may make alterations within a product. polyketide, particularly by replacing an extension module by one that gives a ketide unit at a different oxidation state and/or with a different stereochemistry. NB: It has generally been assumed that the stereochemistry of the methyl groups in the polyketide chain is determined by the acyltransferase. But it is in fact a feature of other domains of the PKS, and thus open to variation only by replacement of those domains, individually or by module replacement. Methyl and other substituents can be added or removed by acyltransferase domain replacement or total module replacement.

This aspect of the invention is largely concerned with treating PKS gene modules as building blocks that can be used to construct enzyme systems, and thus polyketide products, of desired types. This generally involves the cutting out and the assembly of modules and multi-module groupings. It might be assumed that the correct places

for making and breaking intermodular connections would be in the linking regions between modules. However we have found that it is preferable to make cuts and joins actually within domains (i.e. the enzyme-coding portions), close to the edges thereof. The DNA is highly conserved here between all modular PKS's, and this appears to aid in the construction of hybrids that can be transcribed. It also assists in maintaining the spacing of the active sites of the encoded enzymes, which seems to be important. For example in producing a hybrid gene by replacing the ery loading module by an avr loading module, we removed the ery module together with a small amount of the following ketosynthase (KS) domain. The start of the KS domain (well spaced from the active site) is highly conserved and therefore provides a good splicing site. The excised ery module was then replaced by an avr loading module.

In fact when substituting a loading module, it may be desirable to replace not just the loading module domains (generally acyl transferase (AT) and acyl carrier protein (ACP)) but also the KS at the start of the following extension module. Typically the excised loading module would have provided a propionate starter, and the replacement is intended to provide one or more different starters. But propionate may feed in to the KS of the extension module from a propionate pool in the host cell, leading to dilution of the desired products. This can be largely prevented by substituting an extended loading

module including all or most of the KS domain. (The splice site may be in the end region of the KS gene, or early in the following AT gene.)

When replacing "modules", we are not restricted to 5 "natural" modules. For example a "combinatorial module" to be excised and/or replaced and/or inserted may extend from the corresponding domain of two natural-type modules, e.g. from the AT of one module to the AT of the next, or from KS to KS. The splice sites will be in corresponding 10 conserved marginal regions. A combinatorial module can also be a 'double' or larger multiple, for adding 2 or more modules at a time.

The invention further provides such gene assemblies, vectors containing such gene assemblies, and transformant 15 organisms that can express them. Transformant organisms may harbour recombinant plasmids, or the plasmids may integrate. A plasmid with an *int* sequence will integrate into a specific attachment site (*att*) of a host's chromosome. Transformant organisms may be capable of 20 modifying the initial products, e.g. by carrying out all or some of the biosynthetic modifications normal in the production of erythromycins (as shown in Fig 2B) and/or other polyketides. Use may be made of mutant organisms such that some of the normal pathways are blocked, e.g. to 25 produce products without one or more "natural" hydroxy-groups or sugar groups. The invention further provides novel polyketides as producible, directly or indirectly, by transformant organisms. (This includes polyketides



which have undergone enzymic modification in the organisms and/or have been isolated and subjected to chemical modification.)

In a second aspect the invention provides a hybrid  
5 gene assembly comprising structural gene components operably linked to a promoter which is not naturally linked thereto and is of a type II PKS, preferably linked to its specific cognate activator gene. Particularly preferred is the use of the act I promoter and the Act II-  
10 orf4 activator gene from S.coelicolor, for expression in hosts other than S.coelicolor (usually other actinomycetes, particularly other streptomycetes). The structural gene components may be of a type I PKS gene system.

15 The invention in its second aspect further provides vectors containing such gene assemblies, and transformant organisms that can express them. It is possible to combine the two aspects of the invention, so that a hybrid type I gene is expressed under the control of a type II  
20 promoter.

In a further aspect the invention provides novel polyketides obtainable by means of the previous aspects. These include the following.

- (i) An erythromycin analogue (being a macrolide  
25 compound with a 14-membered ring) in which C-13 bears a side-chain other than ethyl, generally an  $\alpha$ -branched  $C_2$ - $C_5$  alkyl group, a  $C_3$ - $C_8$  cycloalkyl or cycloalkenyl group (optionally substituted e.g. with one or more hydroxy,  $C_{1-4}$

alkyl or alkoxy groups or halogen atoms), or a 3-6 membered heterocycle containing O or S, saturated or fully or partially unsaturated, optionally substituted (as for cycloalkyl). Preferred candidates for the C-13 substituent R are the groups of carboxylate units R. C0.0-usable as substrates by an avr starter module, or rapamycin starter variants. Preferred substrates include isobutyrate (R=i-Pr) and 2-methylbutyrate (R=1-methylpropyl). Other possibilities include n-butyrate, cyclohexyl carboxylate, cycloheptanyl carboxylate, cyclohexenyl carboxylates, cycloheptenyl carboxylates, and ring-methylated variants of the cyclic carboxylates.

The erythromycin analogue may correspond to the initial product of a PKS (6-deoxyerythronolide) or the product after one or more of the normal biosynthetic steps. As shown in Fig. 2B these comprise: 6-hydroxylation; 3-O-glycosylation; 5-O-glycosylation; 12-hydroxylation; and specific sugar methylation.

Thus the analogues include ones corresponding to 6-deoxyerythronolide B, erythromycin A, and the various intermediates and alternatives shown in Fig. 2B.

Additionally or alternatively, there may be chemical modification. For example one or more hydroxy groups may be oxidised (e.g. to produce 3-keto derivatives) or eliminated (e.g. to produce 10-ene derivatives). Some examples of chemical modifications applicable to the present inventions are those that give rise to azithromycin, roxithromycin, clarithromycin and those

disclosed in some French patents of Roussel Uclaf:2697523, 2697524 and 2702480.

(ii) erythromycin analogues differing from the corresponding 'natural' compound (Figure 2b) in the oxidation state of one or more of the ketide units (i.e. selection of alternatives from the group:  $-CO-$ ,  $-CH(OH)-$ ,  $-CH-$ , and  $-CH_2-$ ).

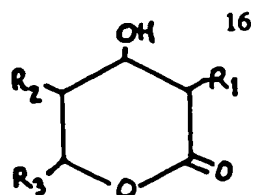
The stereochemistry of any  $-CH(OH)-$  is also independently selectable.

10 (iii) erythromycin analogues differing from the corresponding 'natural' compound in the absence of a 'natural' methyl side-chain. (This is achievable by use of a variant AT). Normal extension modules use either  $C_2$  or  $C_3$  units to provide unmethylated and methylated ketide 15 units. We may provide unmethylated units where methylated units are natural (and vice versa, in systems where there are naturally unmethylated units) and also provide larger units, e.g.  $C_4$  to provide ethyl substituents.

(iv) erythromycin analogues differing from the 20 corresponding 'natural' compound in the stereochemistry of 'natural' methyl; and/or ring substituents other than methyl.

(v) erythromycin analogues having the features of two or more of sections (i) to (iv);

25 (vi) triketide lactone ("TKL") analogues:

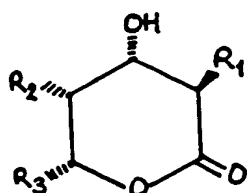


(I)

R<sub>1</sub> is the side-chain derived from the starter unit, 5 and is subject to the variation described for the C-13 sidechain described above in (i).

R<sub>2</sub> and R<sub>3</sub> are "naturally" methyl but either or both may be replaced by hydrogen or ethyl (using extender units employing butyrate)

10 The natural stereochemistry is



(II)

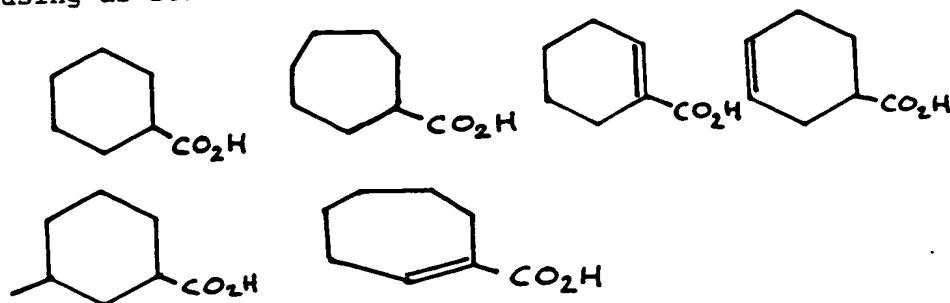
15

but any one or two or all of R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub> and OH may have the opposite stereochemistry. Generally TKL analogues can have variations as described for erythromycins in (i) to (v) above.

20 (vi) polyketides of types other than erythromycin, e.g. rapamycin or avermectin, having modifications corresponding to those described in sections (i) to (v).

For example, we have produced rapamycin variants using as starters:

25



viii) truncated or extended versions of polyketide chains:

a) diketides  $R^1\text{-CHOH-CHR}^2\text{-CO}_2\text{H}$

b) triketides  $R^1\text{-CHOH-CHR}^2\text{-CHOH-CHR}^3\text{-CO}_2\text{H}$

c) tetraketides  $R^1\text{-CHOH-CHR}^2\text{-CHOH-CHR}^3\text{-CHOH-CHR}^4\text{-CO}_2\text{H}$

5 d) penta-, hexa-, hepta- and larger ketide chains

The chains may have variants as described in (i) to (iv).

ix) ketide/non-ketide fusions.

Rapamycin is a natural example of a polyketide/peptide  
10 fusion. Means such as a peptide incorporating enzyme may  
be employed to create polyketides fused to one or more  
amino acids.

x) Polyketides (or fusions) cyclised by formation of  
15 lactones, hemiketals, ketals, lactams, or lactols.

xi) derivatives of any of the above which have undergone  
further processing by non-PKS enzymes, eg one or more of  
hydroxylation, epoxidation, glycosylation, and  
20 methylation.

The present invention provides a method of obtaining  
novel complex polyketides; and novel methods of increasing  
production of both new and known polyketides.

Thus in one type of embodiment of the invention, one  
25 or more segments of DNA encoding individual modules or  
domains within a natural Type I PKS (the "donor" PKS) have  
been used to replace the DNA encoding, respectively,  
individual modules or domains of another natural Type I

PKS (the "acceptor" PKS). The total number of extension modules assembled in the hybrid PKS is not fixed, but the preferred number of such modules in any one multienzyme or cassette ranges between one, creating the smallest 5 possible functional PKS, and six, which equals the largest number of consecutive modules found to be housed in a single multienzyme of a natural Type I PKS, namely the rap PKS of *Streptomyces hygroscopicus*.

In a particularly preferred embodiment for the 10 purposes of defining which hybrid PKS genes will be viable and productive, the acceptor PKS DNA consists of, or comprises of, the loading module, first two extension modules and chain-terminating thioesterase of the ery PKS, or other, preferably natural, type I PKS, housed in a 15 suitable plasmid vector. Either one or more individual domains, or one or more individual modules, are specifically replaced by DNA encoding analogous domains or modules and derived from a different natural Type I PKS (the "donor" PKS). The altered DNA sequence is introduced 20 into a suitable microorganism and the genetically engineered microorganism is cultured under conditions suitable for polyketide production.

Surprisingly and unexpectedly, these genetically engineered microorganisms when cultured under suitable 25 conditions have been found to produce non-natural analogues of the polyketide product(s) of the natural acceptor PKS, and where appropriate the products are found to undergo the same processing as the natural polyketide.

In this aspect of the invention, the plasmid vector may be any one drawn from a long list of plasmid vectors well known to be useful for cloning in *Streptomyces* and related Gram positive bacteria. It has been found particularly useful to select a low copy number plasmid vector with a broad host range based on the SCP2\* plasmid of *Streptomyces coelicolor* M110. The construction is described herein of two SCP2\*-derived plasmids particularly suitable for this purpose. The triketide lactone synthase of the "acceptor" PKS may be composed of loading modules, extension modules and chain-terminating activities drawn from any natural or non-natural Type I PKS, but particularly suitable for this purpose are the components of Type I PKSs for the biosynthesis of erythromycin, rapamycin, avermectin, tetroneasin, oleandomycin, monensin, amphotericin and rifamycin, for all of which the gene and modular organisation is known through gene sequence analysis, at least in part. Particularly favourable examples of the loading modules of the donor PKS are those loading modules showing a relaxed specificity, for example the loading module of the avermectin (avr)-producing PKS of *Streptomyces avermitilis*; or those loading modules possessing an unusual specificity, for example the loading modules of the rapamycin-, FK506- and ascomycin-producing PKSs, all of which naturally accept a shikimate-derived starter unit.

Genetically-engineered cells suitable for expression

of hybrid Type I PKS genes may be drawn from any actinomycete capable of maintaining the vector in either autonomous or integrated form. Particularly effective hosts are *Saccharopolyspora erythraea*, *Streptomyces coelicolor*, *Streptomyces avermitilis*, *Streptomyces griseofuscus*, *Streptomyces cinnamonensis*, *Micromonospora griseorubida*, *Streptomyces hygroscopicus*, *Streptomyces fradiae*, *Streptomyces longisporoflavus*, *Streptomyces lasaliensis*, *Streptomyces tsukubaensis*, *Streptomyces griseus*, *Streptomyces venezuelae*, *Streptomyces antibioticus*, *Streptomyces lividans*, *Streptomyces rimosus* and *Streptomyces albus*. These include hosts in which SCP2\*-derived plasmid vectors are known to replicate autonomously, as for example *S. coelicolor*, *S. avermitilis* and *S. griseofuscus*; and other hosts such as *Saccharopolyspora erythraea* in which SCP2\*-derived plasmids become integrated into the chromosome through homologous recombination between sequences on the plasmid insert and on the chromosome.

20 In a further aspect of the present invention, a plasmid containing "donor" PKS DNA is introduced into a host cell under conditions where the plasmid becomes integrated into an acceptor PKS genes on the bacterial chromosome by homologous recombination, to create a hybrid  
25 PKS. A preferred embodiment is when the donor PKS DNA includes a segment encoding a loading module, in such a way that this loading module becomes linked to the acceptor PKS genes on the chromosome. Such a hybrid PKS



produces valuable and novel hybrid polyketide products when cultured under suitable conditions as described herein. Specifically, when the loading module of the acceptor PKS is replaced by the loading module of the 5 avermectin-producing (avr) PKS, the hybrid polyketide products contain a starter unit typical of those used by the avr PKS. Thus when the loading module of the ery PKS is replaced by the avr loading module, *Saccharopolyspora erythraea* strains containing such hybrid PKS are found to 10 produce 14-membered macrolides containing starter units typically used by the avr PKS.

It is very surprising and unexpected that the 14-membered macrolide polyketides produced by such recombinant cells of *S. erythraea* are found to include 15 derivatives of erythromycin A, showing that the several processing steps required for the transformation of the products of the hybrid PKS into novel and therapeutically valuable erythromycin A derivatives are correctly carried out.

20 A further aspect of the present invention is the unexpected and surprising finding that transcription of any of the hybrid Type I PKS genes, whose construction is described herein, can be specifically increased when the hybrid genes are placed under the control of a promoter 25 for a Type II PKS gene linked to a specific activator gene for that promoter. It is particularly remarkable that when a genetically engineered cell containing hybrid Type I genes under such control is cultured under conditions

suitable for polyketide production, significantly enhanced levels of the hybrid polyketide are produced. Such specific increases in yield of a valuable polyketide product are also seen for natural polyketides produced by a Type I PKS placed under the control of a Type II PKS promoter and activator gene. In a preferred embodiment, Type I PKS genes present on an SCP2\*-derived plasmid are placed under the control of the bidirectional actI promoter derived from the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor*, and in which the vector also contains the structural gene encoding the specific activator protein Act II-orf 4. The recombinant plasmid is introduced into bacterial hosts other than *Streptomyces coelicolor* chosen from *Streptomyces* and related genera, under conditions where either the introduced PKS genes, or PKS genes already present in the host strain, are expressed under the control of the actI promoter.

The recombinant strains produce the desired specific polyketide product and the activator gene requires only the presence of the specific promoter in order to enhance transcriptional efficiency from the promoter. This is particularly surprising in that activators of the ActII-orf4 family do not belong to a recognised class of DNA-binding proteins. Therefore it would be expected that additional proteins or other control elements would be required for activation to occur in a heterologous host not known to produce actinorhodin or a related

isochromanequinone pigment. It is also surprising and useful that the recombinant strains produce up to 10-fold more specific polyketide product than when the same PKS genes are under the control of the natural promoter, and the specific polyketide product is also produced precociously in growing culture, rather than only during the transition from growth to stationary phase. Such polyketides are useful as antibiotics, anti-cancer agents, immunosuppressants and for many other purposes in human and veterinary medicine.

When the genetically engineered cell is *Saccharopolyspora erythraea*, the activator and promoter are derived from the actinorhodin PKS gene cluster and the actI/actII-orf4-regulated ery PKS gene cluster is housed in the chromosome, following the site-specific integration of a low copy number plasmid vector, culturing of these cells under suitable conditions produces up to ten fold more total 14-membered macrolide product than in a comparable strain not under such heterologous control.

When in such a genetically engineered cell of *S. erythraea* the PKS genes under this heterologous control are hybrid Type I PKS genes whose construction is described herein, then again up to ten-fold more hybrid polyketide product is obtained compared to the same hybrid Type I PKS genes not under such control. Specifically, when the hybrid Type I PKS genes are the ery PKS genes in which the loader module is replaced by the avr loading module, a ten-fold increase is found in the total amounts of novel

14-membered macrolides produced by the genetically engineered cells when cultured under suitable conditions as described herein.

The suitable and preferred means of growing the 5 genetically engineered cells, and the preferred means of isolating both the natural and the hybrid polyketides are described more fully in the Examples.

Some embodiments of the invention will now be described with reference to the accompanying drawings in 10 which:

Fig. 1 gives the chemical formulae of three known polyketides;

Fig. 2A is a diagram showing the functioning of 6-deoxyerythronolide synthase B (DEBS), a PKS producing 6-15 deoxyerythronolide B (6-DEB), a precursor of erythromycin A;

Fig. 2B shows post-PKS biosynthesis of erythromycins including the conversion of 6-DEB to erythromycin A;

Fig. 3 is a diagram showing the biosynthesis of 20 rapamycin;

Fig. 4 is a diagram showing the construction of plasmid pDEL702;

Fig. 5 is a diagram showing the construction of plasmid pCJR101;

25 Fig. 6 is a diagram showing the construction of plasmid pCJR110;

Fig. 7 is a diagram showing the construction of plasmid pNTEP2;

Fig. 8 is a diagram showing the construction of plasmid pRMTE and pCJRTE;

Fig. 9 a and b is a diagram showing the construction of plasmid pIG1;

5 Fig. 10 is a diagram showing the construction of plasmid pKW15; this includes DNA encoding a loading module, a first extender module, and the chain-terminating thioesterase, capable of receiving modules;

Fig. 11 is a diagram showing the construction of  
10 plasmid pAR33;

Fig. 12 is a diagram showing the construction of plasmid pAR8;

Fig. 13 is a diagram showing the construction of plasmid pE1A2TE;

15 Fig. 14 is a diagram showing the construction of plasmid pMO7;

Fig. 15 is a diagram showing the construction of plasmid pAVLD;

Fig. 16 shows the integration of pAVLD into the  
20 genome of *S. erythraea* NRRL2338; and

Fig. 17 shows the integration of pAVLD into the genome of *S. erythraea* TER43.

The present invention will now be illustrated, but is not intended to be limited, by means of some examples.

25 Use was made of the following media and solutions.

Sucrose-Succinate defined medium

sucrose	69g
KNO <sub>3</sub>	10g

26

succinic acid	2.36g
KH <sub>2</sub> PO <sub>4</sub>	2.7g
MgSO <sub>4</sub> .7H <sub>2</sub> O	1.2g
ZnCl <sub>2</sub>	10mg
5 MnCl <sub>2</sub> .4H <sub>2</sub> O	6.2g
CuCl <sub>2</sub> .2H <sub>2</sub> O	0.53mg
CoCl <sub>2</sub>	0.55mg
FeSO <sub>4</sub> .7H <sub>2</sub> O	2.5mg
CaCl <sub>2</sub> .2H <sub>2</sub> O	38mg
10 milli-Q water	to 11
KOH	To pH 6-6.4

YEME

sucrose	340g
yeast extract	3g
15 peptone	5g
malt extract	3g
glucose	10g
water	to 1.0l

Tap water medium

glucose	5g
tryptone	5g
yeast extract	2.5g
EDTA	36mg
tap water	to 1.0 l
KOH	to pH7.1

after sterilisation: 2.5M MgCl<sub>2</sub>, 2ml

20

Trace elements solution: ZnCl<sub>2</sub>, 40mg/l; FeCl<sub>3</sub>.6H<sub>2</sub>O, 200mg/l; CuCl<sub>2</sub>.2H<sub>2</sub>O, 10 mg/l; MnCl<sub>2</sub>.4H<sub>2</sub>O, 10mg/l; Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>.10H<sub>2</sub>O, 10mg/l; (NH<sub>4</sub>)<sub>6</sub>MO<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O, 10mg/l; 4mg/ml lysozyme,

BW1 medium

25 CaCO <sub>3</sub>	2 g
Difco tryptone	2.5 g
soy flour	5 g
Difco yeast extract	5 g

27

soluble starch (Sigma) 20 g

pH 7.2

5 $K_2HPO_4$	1.2 g
$MgSO_4 \cdot 7H_2O$	1.2 g
$FeSO_4 \cdot 7H_2O$	0.012 g
$MnSO_4$	0.0012 g
$ZnSO_4 \cdot 7H_2O$	0.0012 g
10 Tap water	to 1.0 l

BW2 Medium

$CaCO_3$	7 g
soy flour	5 g
Difco yeast extract	5 g
15 soluble starch (Sigma)	80 g
$K_2HPO_4$	1 g
$MgSO_4 \cdot 7H_2O$	1 g
10 ml of a trace elements solution as for tap water medium.	

20

Made up to 1 litre with distilled water. pH adjusted to 7.2.

Example 1

25 Construction of strain *Saccharopolyspora erythraea* JC2

An *S. erythraea* host cell, genetically engineered to remove all of the native *eryA* genes which encode the

erythromycin-producing type I PKS, except for the region of eryAIII DNA encoding the chain-terminating thioesterase, was constructed by homologous recombination starting from *S. erythraea* NRRL2338. *S. erythraea* NRRL2338 is a wild-type erythromycin-producing strain obtained from the Northern Regional Research Laboratories, Peoria, Illinois, USA, under the above designation. The ery cluster is made up of the PKS genes, flanked by other genes involved in later stages of erythromycin biosynthesis, including those involved in glycosylation, hydroxylation and methylation.

Plasmid pDEL was constructed as follows (Figure 4). The 1.4 kbp SmaI segment containing the start codon of eryAI was cloned into pUC18 to give p612SL, the segment was excised as a BamHI-SacI fragment using the multiple cloning sites of pUC18, and subcloned into a derivative of plasmid pT7-18 (Roberts, G. A. et al. Eur. J. Biochem. (1993) 214:305-311) containing the SacI/KpnI fragment of eryAIII that encodes the C-terminus of DEBS3 from which a BglII-SacI fragment had been excised. The identity of plasmid pDEL was confirmed by restriction analysis.

Plasmid pDEL was digested with BglII and treated with calf intestinal alkaline phosphatase, and ligated to plasmid pIJ702 (Katz, E. et al. J. Gen. Microbiol. (1983) 129:2703-2714) which had been linearised with BglII. The resulting mixture contains the desired plasmid pDEL702.

Protoplasts of *S. erythraea* NRRL2338 (Yamamoto, H. et al. J. Antibiot. (1986) 39:1304-1313) were transformed



with 10 µg pDEL702 and stable thiostrepton resistant colonies were isolated. Individual colonies were selected and subcultured four times in non-selective liquid medium (tryptone soy broth) followed by preparation and 5 regeneration of protoplasts. Thiostrepton sensitive colonies were isolated and characterised by restriction analysis and Southern hybridisation. One such colony was designated JC2. *S. erythraea* strain JC2 has been deposited at the National Collection of Industrial and 10 Marine Bacteria, 23 St Machar Drive, Aberdeen, Scotland AB2 1RY, under the designation NCIMB 40802.

#### Example 2

##### 15 Construction of the Recombinant Vector pCJR101

pCJR101 (Figure 5) is a shuttle plasmid constructed to be used for expression of PKS genes in actinomycetes. It includes a ColEI replicon to allow it to replicate in *E. coli*, an SCP2\* low copy number *Streptomyces* replicon 20 (Bibb, M. J. and Hopwood, D. A. J. Gen. Microbiol. (1981) 126:427) and the actII-orf4 activator gene from the act cluster which activates transcription from the act promoter during the transition from growth phase to 25 stationary phase in the vegetative mycelium. It is constructed as follows: an approximately 970 bp DNA fragment from pMF1015 (containing the actII-orf4 activator gene) (Fernandez-Moreno, M. A. et al. Cell (1991)

66:769-780) is amplified by PCR, using as primers the synthetic oligonucleotides:

5'-ACT AGT CCA CTG CCT CTC GGT AAA ATC CAG C-3' and 5'-CTT AAG AGG GGC TCC ACC GCG TTC ACG GAC-3', which also introduces flanking SpeI and AflII restriction sites. This fragment is end-repaired and introduced by blunt-ended ligation into the AatII site of plasmid pUC19 to yield plasmid p18.14. An approximately 215 bp DNA fragment is amplified from pMV400 which contains the bidirectional promoter pair PactIII/PactI (Parro, V. et al. Nucl. Acids Res. (1991) 19:2623-2627), using as primers the synthetic oligonucleotides 5'-ACA TTC TCT ACG CCT AAG TGT TCC CCT CCC TGC CTC-3' and 5'-GTG ATG TAT GCT CAT ATG TGT CCT CCT TAA TTA ATC GAT GCG TTC GTC CGG TG-3', which also introduces flanking NdeI and AflII sites. The PCR product is digested with NdeI and AflII and ligated with the plasmid p18.14 previously cut with NdeI and AflII, to generate plasmid p19.4. A 1.1 kbp HindIII-SphI fragment containing the *tsr* gene, which confers resistance to thiostrepton, is obtained by PCR from plasmid pIJ922 (Lydiate, D. J. et al. Gene (1985) 35:223-235) as template, using as primers the oligonucleotides 5'-TGA ACA CCA AGC TTG CCA GAG AGC GAC GAC TTC CCC-3' and 5'-GAC AGA TTG CAT GCC CTT CGA GGA GTG CCC GCC CGG-3' which also introduces flanking HindIII and SphI sites. The PCR product is digested with HindIII and SphI and ligated with plasmid p19.4 cut with HindIII and SphI to obtain plasmid p20.5. The plasmid pIJ922 is digested with BamHI and SstI

and the fragment containing a portion of the fertility locus and the origin of replication (Lydiate, D. J. et al. Gene (1985) 35:223-235) is ligated into pUC19 digested with BamHI and Sst I to generate the bifunctional plasmid 16/2.2 (14.7 kbp). Plasmid 20.5 is digested with SalI and SphI, the two larger fragments from the digest are purified by gel electrophoresis, and combined in a four-component ligation with plasmid 16/2.2 which has been digested with XhoI and SphI. The ligation mixture is used to transform *Streptomyces lividans* and colonies are selected in the presence of thiostrepton. One such colony is shown to contain the desired plasmid pCJR101 (approx. 12.4 kbp), identified by its restriction pattern.

15

## Example 3

## Construction of plasmid pCJR110

The construction of plasmid pCJR110 is illustrated in Figure 6. A 1.1 kbp HindIII-XhoI fragment containing the *tsr* gene, which confers resistance to thiostrepton, is obtained by PCR from plasmid pIJ922 as template, using as primers the oligonucleotides 5'-TGA ACA CCA AGC TTG CCA GAG AGC GAC GAC TTC CCC-3' and 5'-GAC AGA TTC TCG AGC CTT CGA GGA GTG CCC GCC CGG-3' which also introduces flanking HindIII and XhoI sites. The PCR product is digested with HindIII and XhoI and ligated with plasmid 16/2.2 which has been digested with HindIII and XhoI, to generate plasmid

22.1. Plasmid 22.1 is digested with HindIII and SphI and ligated with plasmid 19.4 which has been digested with HindIII and SphI, to produce the desired plasmid pCJR110 (approx. 12.4 kbp), identified by its restriction pattern.

5 Plasmid pCJR110 differs from pCJR101 in the orientation of the *tsr* gene, the *actII-orf4* gene and the *actI/actIII* promoter, with respect to the SCP2\*-derived origin of replication.

10

#### Example 4

#### Construction of plasmid pRM52

Plasmid pRM52 is a derivative of plasmid pRM5 (McDaniel, 15 R. et al. Science, (1993) 262:1546-1550). pRM5 was first linearised by digestion with NdeI, end-repaired and then religated to produce pRM51. pRM51 was cut with PacI and NsiI and the large PacI-NsiI fragment was isolated and ligated to a short double-stranded oligonucleotide linker 20 containing an NdeI site and constructed from the synthetic oligonucleotides 5'-TAAGGAGGACACATATGCA-3' and 5'-TAATTCCTCCTGTGTAT-3' which were annealed together. The ligation mixture was transformed into E. coli TGIrecO and isolated colonies were screened for their plasmid content. 25 The desired plasmid (19.6 kbp) was identified by its restriction map and was designated pRM52.

## Example 5

## Construction of plasmid pNTEP2

Plasmid pNTEP2 contains the entire open reading frame for the chimaeric DEBS1 plus thioesterase gene, with a unique NdeI site at the start codon and unique XbaI and HindIII sites immediately 3' of the stop codon. It is constructed via several intermediate plasmids as follows (Figure 7):

## 10 Construction of plasmid pTENC011

A library of total DNA from *S. erythraea* TED8 (Cortés, J. et al. Science (1995) 268: 1487-1489) was constructed in the vector  $\lambda$ DASH II (Stratagene) and probed with *eryA* gene fragments. One recombinant bacteriophage designated  $\lambda$ -4B had an insert extending from 700 bp upstream of the *eryAI* start codon to the thiostrepton resistance gene of the integrated plasmid in *S. erythraea* TED8.

The  $\lambda$ -4b DNA was digested with NcoI and the 12 kbp NcoI fragment was end-repaired and ligated into SmaI-cut pUC18 and transformed into *E. coli* TG1recO. Individual colonies were screened for their plasmid content and one plasmid bearing the NcoI insert was selected and designated pTENC011.

## 25 Construction of plasmid pNK8

A 4.0 kb KpnI fragment extending from 1.4 kbp upstream of the correct *eryAI* start codon as previously determined (Caffrey, P. et al. FEBS Letters (1992) 304:225-228), to

2.6 kbp inside the eryAI gene of *S. erythraea*, was excised from plasmid pEK25 (Revitt, D. J. et al. Eur. J. Biochem. (1992) 204:39-49) and cloned into pTZ18R to obtain plasmid pBK6.12. DNA of this plasmid was used as  
5 the template for a PCR reaction to obtain a 360 bp product in which a unique Nde I site is created at the start codon of eryAI and a unique SmaI site is created at the other end of the PCR product. The oligonucleotides used were 5'-CCC ATA TGG CGG ACC TGT CAA AGC-3' and 5'-ATT GCG CGC  
10 CCT GGC CCC GGA A-3'. The product was end-repaired and ligated into SmaI cut pUC18, and transformed into *E. coli* TG1recO.

Individual colonies were screened for their plasmid content and one plasmid bearing the insert in an  
15 orientation such that the SmaI site was adjacent to the KpnI site of the polylinker was selected and designated plasmid pNDE6. Plasmid pNDE6 was digested with SmaI and KpnI, and ligated with a 3 kbp fragment of the eryAI gene obtained by digestion of plasmid pBK6.12 with SmaI  
20 and KpnI. The ligation mixture was used to transform *E. coli* TG1recO and individual colonies were screened for their plasmid content. A plasmid containing the desired 2.6 kbp NdeI-KpnI fragment was isolated and designated plasmid pNDE7. The NdeI-KpnI insert was excised from  
25 plasmid pNDE7 and ligated into plasmid pT7-18, previously digested with NdeI and KpnI. Plasmid pT7-18 is a derivative of plasmid pT7-7 (Tabor, S. and Richardson, C.C. Proc. Natl. Acad. Sci. USA (1985) 82:1074-1078) in

which the polylinker is replaced by the polylinker from pUC18. The ligation mixture was used to transform *E. coli* TG1recO and individual colonies were screened for their plasmid content and one plasmid containing the desired 2.6 kbp NdeI-KpnI insert was selected and designated pNK8.

#### Construction of plasmid pNTE5

Plasmid pNK8 was transformed into a methylation-deficient strain of *E. coli* ET12567 (MacNeil, D.J. et al. Gene (1992) 111:61-68) and the plasmid pNK8 was isolated from this strain and digested with ClaI. An 11 kbp ClaI fragment obtained by digestion of pTENC011 was ligated into the digested pNK8 and transformed into *E. coli* TG1recO. Individual colonies were screened for their plasmid content and one plasmid, in which the 11 kbp insert was correctly oriented to regenerate the reading frame of *eryAI*, was selected and designated pNTE5.

#### Construction of plasmid pNTEP2

A ClaI-EcoRI polylinker, bearing unique restriction sites for XbaI and for HindIII was constructed, from the following complementary synthetic oligonucleotides:

5'-AATTCATAGTCTAGAAGCTTAT-3'

and

5'-CGATAAGCTTCTAGACTATG-3'

The polylinker was ligated into plasmid pNTE5, which had been digested with ClaI and EcoRI to remove a 2.3 kbp ClaI-EcoRI fragment. The ligation mixture was used to transform *E. coli* TG1recO and individual colonies were screened for their plasmid content. One plasmid

containing the polylinker was identified and designated pNTEP2.

#### 5 Example 6

##### Construction of plasmid pRMTE

Plasmid pNTEP2 (14 kbp) was digested with NdeI and XbaI and the insert was purified by sedimentation on a sucrose  
10 gradient. The purified insert was ligated into plasmid pRM52 (19.6 kbp) (Example 4) which had been digested with NdeI and XbaI, and the vector purified by sedimentation on a sucrose gradient. The ligation mixture was used to  
15 transform E. coli and individual colonies were checked for their plasmid content. The desired plasmid pRMTE (31.5 kbp) was identified by its restriction pattern (Figure 8).

#### Example 7

##### 20 Construction of plasmid pCJRTE

Plasmid pNTEP2 (Example 5) is digested with NdeI and XbaI and the insert is purified by sedimentation on a sucrose  
gradient. The purified insert is ligated into plasmid  
25 pCJR101 (12.4 kbp) which has been digested with NdeI and XbaI, and purified by sedimentation on a sucrose gradient. The ligation mixture is used to transform E. coli DHB10 and individual colonies are screened for their plasmid



content. The desired plasmid pCJRTE (24.3 kbp) is identified by its restriction pattern (Figure 8).

#### Example 8

5 Construction of *S. avermitilis* ATCC 31272/pCJRTE and production of triketide lactone ("TKL") derivatives therewith.

##### (i) Construction

Approximately 5  $\mu$ g of plasmid pCJRTE is transformed into  
10 protoplasts of *S. avermitilis* ATCC 31272 and stable thiostrepton resistant colonies are isolated. Several such colonies are analysed for their content of plasmid DNA. A colony containing a plasmid whose restriction map shows it to be identical to pCJRTE, is designated S.  
15 *avermitilis* ATCC 31272/pCJRTE.

##### (ii) Production of (Ac)-TKL and TKL

*S. avermitilis* ATCC 31272/pCJRTE is inoculated into medium  
20 BW1 containing 50  $\mu$ g/ml thiostrepton, and allowed to grow for four days at 28-30°C. After this time, 15 ml of the cell suspension is used to inoculate 150 ml of liquid medium BW2 containing 50  $\mu$ g/l thiostrepton, and allowed to grow for 6 days. After this time the cells are removed by  
25 centrifugation, washed with water, and the supernatants are combined and extracted three times with ethyl acetate (250 ml). The combined ethyl acetate extracts are washed with an equal volume of saturated sodium chloride, dried

over anhydrous sodium sulphate and the ethyl acetate is removed by evaporation under reduced pressure. Samples of the residue are taken up in a minimal quantity of diethyl ether, filtered through a plug of silica, and analyzed by GC, which reveals the presence of both (Ac)-TKL and TKL (Formula II:  $R_1=R_2=Me$ ;  $R_3=Me$  for (Ac)-TKL, and Et for TKL), with identical retention times to authentic synthetic samples.

#### 10 Example 9

##### Construction of plasmids pIG1 and pIG101

Plasmids pIG1 and pIG101 each consist of an SCP2\*-derived plasmid containing a hybrid Type I PKS gene comprising the avr loading module in place of the ery loading module, the first two extension modules of the ery PKS and the thioesterase of the ery PKS. These are constructed via several intermediate plasmids as follows (Figure 9).

#### 20 Construction of plasmid pVE 3.4

Plasmid pVE1446 which contains a portion of the avermectin (avr) PKS genes was obtained from E. coli strain ATCC 68250 (MacNeil, D. J. et al. Ann. N. Y. Acad. Sci. (1994) 721:123-132). Plasmid pVE1446 was digested with BamHI and the 7.6 kbp fragment between coordinates 32.15 and 3.40 (MacNeil, D. J. et al. Ann. N. Y. Acad. Sci. (1994) 721:123-132) was purified by gel electrophoresis and

recircularised. The mixture contained the desired plasmid pVE3.4 which was isolated after transformation of *E. coli* strain TG1recO.

#### 5 Construction of plasmid pNCO12

Plasmid pBK25 (Bevitt, D. J. et al. *Eur. J. Biochem.* (1992) 204:39-49) was digested with NcoI and the 12 kbp fragment was end-repaired and ligated into plasmid pUC18 which had been linearised with SmaI. The ligation mixture was transformed into *E. coli* TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pNCO12 was identified by its restriction pattern.

15

#### Construction of plasmid pCRabc

Plasmid pCRabc (Figure 9) was constructed as follows. Three separate PCR reactions were conducted: First, 20 pmol each of synthetic oligonucleotides A1 (5'-CTC GTC GGT GGC TTT GCG-3') and A2 (5'-CCC GGG AAA AAC GAA GAC TAG TGG CGC GGA CGG CCG-3') were used to amplify a 1.0 kbp product from 100 ng pNCO12 template. The PCR product was end-repaired, phosphorylated and cloned into SmaI-cut pUC18 to obtain plasmid pCRa. Secondly, 20 pmol each of synthetic oligonucleotides C1 (5'-CAC GCG CAG CGC GGC GGA-3') and C2 (5'-CGAA CCG CTA GCG GTC GTC GCG ATG GCC T-3') were used to amplify a 1.5 kbp product from 100 ng

pNC012 template. The product was end-repaired, phosphorylated and cloned into SmaI-cut pUC18 to obtain plasmid pCRc. Thirdly, 20 pmol each of synthetic oligonucleotides B1

5 (5'-GTGGCCCGGCCGTCCGCGCCACTAGTCTTCGTTTTT-3') and B2 (5'-AAC AGCTAGCGGTTTCGTCCGCGCTGCCGTGCC-3') were used to amplify a 1.4 kbp product from 100 ng pVE3.4 template. The product was end-repaired, phosphorylated and cloned into SmaI-cut pUC18 to obtain plasmid pCRb.

10

Plasmid pCRa was digested with HindIII and SpeI and the 1.0 kbp insert was ligated with plasmid pCRb previously digested with HindIII and SpeI, to obtain plasmid pCRab.

Plasmid pCRc was digested with NheI and EcoRI and the 1.5 kbp insert was ligated with plasmid pCRab previously digested with NheI and EcoRI to obtain plasmid pCRabc.

#### Construction of plasmid pNEWAVETE

20 Plasmid pCRabc was digested with MfeI and SfiI and the DNA fragment containing the loading domain of the avr PKS was purified by gel electrophoresis and ligated with plasmid pNTEP2 which had been digested with MfeI and SfiI and the larger fragment purified by gel electrophoresis. The  
25 ligation mixture was transformed into E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pNEWAVETE (13.7 kbp) was identified by its restriction pattern.

### Construction of plasmid pIG1

Plasmid pNEWAVETE was digested with NdeI and XbaI and the  
5 insert was purified by sedimentation on a sucrose  
gradient. The purified insert was ligated into plasmid  
pRM52 (19.6 kbp) which had been digested with NdeI and  
XbaI, and the vector purified by sedimentation on a  
sucrose gradient. The ligation mixture was used to  
10 transform E. coli and individual colonies were checked for  
their plasmid content. The desired plasmid pIG1 was  
identified by its restriction pattern.

### Construction of plasmid pIG101

15

Plasmid pNEWAVETE is digested with NdeI and XbaI and the  
insert is purified by sedimentation on a sucrose gradient.  
The purified insert is ligated into plasmid pCJR101  
(Example 2) which has been digested with NdeI and XbaI,  
20 and purified by gel electrophoresis. The ligation mixture  
is used to transform E. coli DHB10 and individual colonies  
are screened for their plasmid content. The desired  
plasmid pIG101 is identified by its restriction pattern.

### 25 Example 10

Construction of S. coelicolor CH999/pIG1 and production of  
TKL derivatives.

#### (i) Construction

Plasmid pIG1 which had been isolated from *E. coli* ET12567 (MacNeil. D. J. et al. Gene (1992) 111:61-68) was used to transform protoplasts of *S. coelicolor* CH999 and stable thiostrepton resistant colonies were isolated. Individual colonies were checked for their plasmid content and the presence of plasmid pIG1 was confirmed by its restriction pattern.

(ii) Production of TKL, (Ac)TKL, (i-but)TKL and (s-pent)TKL using *S. coelicolor* CH999/pIG1

*S. coelicolor* CH999/pIG1 was inoculated into 100 ml YEME medium containing 50 µg/ml thiostrepton and allowed to grow for five days at 28-30°C. After this time the broth was filtered to remove mycelia. The broth was extracted three times with quarter volumes of ethyl acetate and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure. The residue was taken up in ethyl acetate and filtered through a plug of silica, the ethyl acetate was again removed and the residue was taken up in diethyl ether and subjected to flash chromatography on a column of silica gel eluted with diethyl ether. A fraction containing (s-pent)-TKL and (i-but)-TKL was separated from a fraction containing TKL, with minor amounts of (Ac)-TKL in a third fraction. The compounds were identified by their co-migration with authentic standards on GC analysis (25m column, programmed for 2

minutes at 70°C, then ramped to 250°C over 24 minutes. The retention times for (s-pent)-TKL, (i-but)-TKL, TKL and (Ac)-TKL were 14.9, 13.6, 12.9 and 11.9 minutes respectively. GC, electrospray MS and <sup>1</sup>H-NMR were used to show that the major component (50-60%) was TKL.

#### Example 11

Construction of *S. coelicolor* CH999/pIG101 and production of TKL derivatives

##### (i) Construction

Plasmid pIG101 which has been isolated from *E. coli* ET12567 (MacNeil, D. J. et al. Gene (1992) 111:61-68) is used to transform protoplasts of *S. coelicolor* CH999 and stable thiostrepton resistant colonies were isolated. Individual colonies are checked for their plasmid content and the presence of plasmid pIG101 is confirmed by its restriction pattern.

20

(ii) Production of TKL, (Ac)TKL, (i-but)TKL and (s-pent)TKL using *S. coelicolor* CH999/pIG101

*S. coelicolor* CH999/pIG101 is inoculated into YEME medium containing 50 mg/ml thiostrepton and allowed to grow for five days at 28-30°C. The broth is extracted three times with quarter volumes of ethyl acetate and the combined ethyl acetate extracts are dried over anhydrous sodium

25

sulphate, and the ethyl acetate is removed under reduced pressure. The residue was treated as in Example 10 and gave similar results.

5

#### Example 12

Construction of *S. avermitilis* ATCC31272/pIG1 and production of TKL derivative

##### (i) Construction

10

Plasmid pIG1 which had been isolated from *E. coli* ET12567 MacNeil, D. J. et al. Gene (1992) 111:61-68) was transformed into protoplasts of *S. avermitilis* ATCC31272 and stable thiostrepton resistant colonies were isolated.

15 Individual colonies were checked for their plasmid content and the presence of plasmid pIG1 was confirmed by its restriction pattern.

##### (ii) Production of TKL, (Ac)TKL, (i-but)TKL and

20 (s-pent)TKL using *S. avermitilis* ATCC31272/pIG1

*S. avermitilis* ATCC31272/pIG1 was first inoculated into medium BW1 containing 50 µg/ml thiostrepton, and allowed to grow for four days at 28-30°C. After this time 20 ml  
25 of the broth is used to seed 150 ml of medium BW2 containing 50µg/ml of thiostrepton.

The inoculated organism was then allowed to grow for 10-12



days. The broth was filtered to remove mycelia, and extracted three times with quarter volumes of ethyl acetate and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure, to give about 10 mg crude product per litre. The residue was dissolved in ethyl acetate, passed through a plug of silica, and the solvent was removed. The residue was dissolved in diethyl ether and subjected to flash chromatography on a silica column (1 cm x 15 cm) eluted with diethyl ether, and fractions of 10 ml each were collected and assayed by GC. The diethyl ether was evaporated to leave about 10 mg of oily residue containing triketide lactones. The major component (50-60%) was (s-pent)-TKL, with (i-but)-TKL, TKL and (Ac)-TKL also present (i.e. compounds of formula II with  $R_1=R_2=Me$ , and  $R_3=1\text{-methylpropyl}$  ((s-pent)-TKL), i-Pr((i-But)-TKL), Et(TKL) and Me((Ac)-TKL).

#### Example 13

20 Construction of *S. avermitilis* ATCC31272/pIG101 and production of TKL derivatives

##### (i) Construction

25 Plasmid pIG101 which has been isolated from *E. coli* ET12567 (MacNeil, D. J. et al. Gene (1992) 111:61-68) is transformed into protoplasts of *S. avermitilis* ATCC31272 and stable thiostrepton resistant colonies are isolated.

Individual colonies are checked for their plasmid content and the presence of plasmid pIG101 is confirmed by its restriction pattern.

- 5 (ii) Production of TKL, (Ac)TKL, (i-but)TKL and (s-pent)TKL using *S. avermitilis* ATCC31272/pIG101

*S. avermitilis* ATCC31272/pIG101 is first inoculated into medium BW1, described above

- 10 and allowed to grow for 10-12 days. Isolation of products as in the previous example gives a fraction containing (s-pent)-TKL and (i-but)-TKL, a fraction containing TKL, and a third fraction with minor amounts of (Ac)-TKL. The compounds are identified by their co-migration with  
15 authentic standards on GC analysis.

#### Example 14

Construction of *S. erythraea* JC2/pIG1 and production of  
20 TKL derivatives

##### (1) Construction

Approximately 5  $\mu$ g of plasmid pIG1 is transformed into  
25 protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies are isolated. From several such colonies, total DNA is obtained and analysed by Southern hybridisation, to confirm that the plasmid has integrated

specifically into the portion of the eryAIII gene that encodes the C-terminal thioesterase/cyclase, by homologous recombination.

5 (ii) Production of triketide lactones using *S. erythraea* JC2/pIG1

*S. erythraea* JC2/pIG1 is inoculated into tap water medium containing 50 µg/ml thiostrepton and allowed to grow for 10 four days at 30°C. After this 20 ml of the mycelium is used to seed 500 ml of sucrose-succinate medium containing 50 µg/ml thiostrepton, in a 2L flask with a single spring to reduce clumping, shaken at 280 rpm. After between 3.5 and 6 days, the broth is filtered to 15 remove mycelia and then extracted three times with a quarter volume of ethyl acetate. The combined ethyl acetate extracts are dried over anhydrous sodium sulphate and solvent removed by evaporation. Analysis of the product mixture using GC and electrospray MS revealed that 20 of a total of 5-6 mg/L of triketide lactone products, the major component was (s-pent)-TKL (about 1.5 mg/L), with other components present being (i-but)-TKL, TKL and a minor amount of (Ac)-TKL.

25 Example 15

Construction of *S. erythraea* JC2/pIG101 and production of TKL derivatives

(i) Construction

Approximately 5 µg of plasmid pIG101 is transformed into protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies are isolated. From several such colonies, total DNA is obtained and analysed by Southern hybridisation, to confirm that the plasmid has integrated specifically into the portion of the *eryA* III gene that encodes the C-terminal thioesterase/cyclase, by homologous recombination.

10

(ii) Production of triketide lactones using *S. erythraea* JC2/pIG101

The same procedure as in Example 14 (ii) was followed. Analysis of the product mixture using GC and electrospray MS revealed that of a total of 5-6 mg/L of triketide lactone products, the major component was (s-pent)-TKL (about 1.5 mg/L), with other components present being (i-but)-TKL, TKL and a minor amount of (Ac)-TKL.

20

Example 16

Construction of plasmid pKW15

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Plasmid pKW15 is a pT7-derived vector containing an insert comprising the loading module, the first extension module and the thioesterase of the *ery* PKS, suitable for

subcloning into an SCP2\*-based vector to obtain expression of a diketide synthase gene; and also suitable for insertion of heterologous DNA containing one or more intact modules. Plasmid pKW15 is obtained via several 5 intermediate plasmids as follows (Figure 10).

#### Construction of plasmid pKW11

Plasmid pNTEP2 (Example 5) was digested with BglIII, the sticky ends were filled in and religated, to produce 10 plasmid pKW11. The insert in plasmid pKW11 consists of a chimaeric eryAI-eryAIII gene encompassing the loading didomain, module 1 and module 2 from DEBS1 and the thioesterase from DEBS3. The strategy to obtain a 'diketide synthase' was to remove the DNA encoding part of 15 module 1, the whole of module 2 and part of the thioesterase, by digestion of plasmid pKW11 with EcoRV and EcoRI, and then to reconstitute module 1 and the N-terminal part of ACP1 by insertion of an appropriate PCR product, and similarly a PCR product was designed to 20 replace the C-terminal part of ACP2 and the thioesterase. The two PCR products are joined by a unique BglIII site created in the active site of the ACP, which involves an alteration in amino acid sequence of the hybrid ACP domain from EL (glutamic acid followed by leucine) as found in 25 both ACP1 and ACP2 domains, to DL (aspartic acid followed by leucine). Such alterations in sequence at a PKS active site, with a view to retaining function, have not been previously attempted and it is not obvious that such

altered sites should remain active.

Construction of plasmids pKW12, pKW13, pKW14 and pKW15

5 For the PCR amplification of DNA for module 1, the following synthetic oligonucleotides were used as mutagenic primers, one containing an EcoRV site and the other a BglII site:

5'-GCAGGGATATCGCACGTTCTCTGG-3'

10 and 5'-CGCCGAGATCTGCGAAGGCCTGGTCGGCGGG-3'

PCR was carried out on pNTEP2 as template using Pfu DNA polymerase and 30 cycles of 95° (1 min); annealing at 55°C (1 min) and extension at 72°C (2 min), in the presence of 15 10% (vol/vol) dimethylsulphoxide. The product (PCR1) was end-repaired and cloned into SmaI-cut phagemid pUC119 and the ligation mixture was used to transform E. coli TG1recO. Plasmid DNA was prepared from individual colonies and the desired plasmid (5.0 kbp) was identified 20 by its restriction pattern and was designated pKW12.

For PCR amplification of the DNA for the 5' end of module 2 and the thioesterase domain, the following oligonucleotides containing respectively a Bgl II site and 25 an EcoRI site, were used as mutagenic primers:

5'-ATGAATTCCTCCGCCAGCCAG-3'

and

5' -ACAGATCTCGGCTTCGACTCGCTGACCG-3'

PCR was carried out on pNTEP2 as template exactly as described above for PCR1 and the product (PCR2) was end-repaired and cloned into SmaI-cut phagemid pUC119. The ligation mixture was used to transform E. coli TG1recO and plasmid DNA was prepared from individual colonies. The desired plasmid (4.1 kbp) was identified by its restriction pattern and was designated pKW13.

10

Plasmid pKW12 was digested with EcoRV and HindIII, and the 1.8 kbp insert was end-repaired, and then ligated together with plasmid pKW11 which had been linearised with EcoRV and treated with alkaline phosphatase. The ligation mixture was transformed into E. coli TG1recO and the plasmid content of individual colonies was checked. The desired plasmid (15.8 kbp) was identified in which the unique Eco RV site had been reconstituted, and this plasmid was designated pKW14.

20

Plasmid pKW13 was digested with BglII and EcoRI and the 0.9 kbp insert was ligated into plasmid pKW14 which had been digested with BglII and EcoRI. The ligation mixture was transformed into E. coli TG1recO and the plasmid content of the individual colonies was checked. The desired plasmid (9.32 kbp) was identified, in which the 0.9 kbp BglII-EcoRI fragment of pKW13 replaced the 9.5 kbp BglII-EcoRI segment of pKW14, and this plasmid was

designated pKW15.

#### Example 17

#### Construction and use of plasmid pKW16

5

##### (i) Construction

Plasmid pKW15 was digested with NdeI and XbaI and the insert was ligated into plasmid pRM52 which had also been  
10 digested with NdeI and XbaI. The ligation was transformed into E. coli TGI recO and isolated colonies were screened for their plasmid content. The desired plasmid was identified by its restriction map and was designated pKW16.

15

##### (ii) Use of plasmid pKW16 for construction of S. coelicolor CH999/pKW16

Plasmid pKW16 was used to transform the  
20 methylation-deficient strain E. coli ET12567 (MacNeil, D. J. et al. Gene (1992) 111:61-68) and the demethylated plasmid pKW16 DNA isolated from this strain was used to transform S. coelicolor CH999 (McDaniel, R. et al. Science (1993) 262:1546-1550. S. coelicolor protoplasts were  
25 transformed with pKW16 and stable thiostrepton resistant colonies were transferred to tap water medium agar plates containing 50 µg/ml thiostrepton.



(iii) Isolation and characterisation of  
(2S)-methyl-(3R)-hydroxypentanoic acid and  
(2S)-methyl-(3R)-hydroxybutanoic acid.

5 A colony of *S. coelicolor* CH999/pKW16 was picked and transferred to 100 ml YEME supplemented with 50 µg/ml thiostrepton and allowed to grow at 30°C. After 4 days the broth was filtered to remove mycelia, acidified to pH 3.0 and solid sodium chloride added until the solution was  
10 saturated. The broth was extracted 5 times with an equal volume of ethyl acetate, and the combined ethyl acetate extracts were dried by extraction with saturated sodium chloride solution and concentrated by evaporation. Thin layer chromatography on silica gel plates, eluted with  
15 ethyl acetate:acetic acid 99:1 (v/v) and stained with potassium permanganate, showed the presence of a compound with the same mobility ( $R_f$  0.55) as a reference sample of (2S)-methyl-(3R)-butanoic acid, which was not present in an extract obtained from *S. coelicolor* CH999 alone.

20 Electrospray mass spectrometry (ESMS) analysis, in the negative ion mode, of the ethyl acetate extracts showed a major peak at  $m/e$  117 not present in the control sample. In positive ion mode, and in the presence of formic acid, a peak was observed at  $m/e$  119, which shifted to  $m/e$  141  
25 in the presence of added sodium ions. The exact mass of the sodium adduct was determined to be 141.05171 (the sodium salt of 2-methyl-3-hydroxybutanoic acid requires 141.05248). When a colony of *S. coelicolor* CH999/pKW16

was picked and transferred to 100 ml YEME supplemented with 50 µg/ml thiostrepton and allowed to grow at 30 °C for 7 days, an ethyl acetate extract prepared as above showed an additional peak, in ESMS operated in negative ion mode, at m/e 131. In ESMS operated in positive ion mode, and in the presence of added formic acid, the peak is found at m/e 155. The exact mass of this peak was determined to be 155.06973 (the sodium salt of 2-methyl-3-hydroxypentanoic acid requires 155.06890).

10

## Example 18

## Construction of plasmid pAR33

Plasmid pAR33 contains a hybrid Type I PKS comprising the 15 ery loading module, extension module 1 of the ery PKS, extension module 12 of the rap PKS, and the ery chain-terminating thioesterase. It is constructed via several intermediate plasmids as follows (Figure 11):

## 20 Construction of plasmid pARRAP

The 4.7 kbp DNA segment of the rapC gene encoding module 12 of the rapamycin PKS was amplified by PCR employing the CloneAmp procedure (Raschtian, A. et al. Anal. Biochem. 25 (1992) 91:91-97) and with the following two oligonucleotides as primers:

5' -ACGCGUACUAGUCAGATCTGGGCATCAATTCGCTGACCGCGGTGGAAGTGGCGAA

-3'

and

5'-AUGGAGAUUCUCUCAGATCTTGAATGCGGCGGCTGCGGGGATGGTGTGGCGTCA-3', and using as template the DNA of clone  $\lambda$ -1C (Schwecke, T. et al. Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843).

5 Approximately 30-60 ng of the PCR product (4.7 kbp) is digested with uracil DNA glycosylase for 30 minutes at 37°C in the presence of 25 ng pAMP18 vector DNA (Gibco BRL), the mixture is cooled on ice and transformed into E. coli TG1recO and individual colonies are checked for their  
10 plasmid content. The desired plasmid (7.4 kbp) is identified by its restriction map and is designated pARRAP.

#### Construction of plasmid pAR32

15

Plasmid pARRAP is digested with BglII to release the 4.7 kbp fragment encoding rap module 12, which is purified by gel electrophoresis and then ligated into plasmid pKW15, which has been linearised by digestion with BglII. The  
20 ligation mixture is transformed into E. coli TG1recO and individual colonies are checked for their plasmid content. The desired plasmid is one in which the rap module 12 has the correct orientation with respect to the coding sequence of the open reading frame of the insert in pKW15,  
25 so that a hybrid triketide lactone synthase gene is produced. Such a plasmid is identified by its restriction pattern, and is designated pAR32.

### Construction of plasmid pAR33

Plasmid pAR32 contains an insert that can be excised by digestion with NdeI and XbaI, but there is an additional NdeI site in the insert that must be specifically protected against cleavage. This is done using the RecA protection method (Koob, M. et al. Nucl. Acids Res. (1992) 20:5831-5835)). The synthetic oligonucleotide 5'-GCACCCACGACGCCACCACCACATATGCCCTGCACCCTGCCCTCC-3' (in which the NdeI site is underlined) is used together with purified RecA protein and ATPγS, to form a stable triplex DNA-protein complex that specifically protects the internal NdeI site in rap module 12 from digestion. The protected plasmid pAR32 is digested with NdeI and XbaI, producing the desired full-length insert (13.1 kbp), and this is ligated with plasmid pRM52 (Example 4) which has been digested with NdeI and XbaI. The ligation mixture is transformed into E. coli TG1 recO and individual colonies are screened for their plasmid content. The desired plasmid pAR33 is identified by its restriction pattern.

#### Example 19

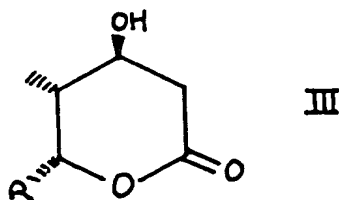
Construction of *S. erythraea* JC2/pAR33 and preparation of TKL derivatives

##### (i) Construction

Approximately 5  $\mu$ g of plasmid pAR33 is transformed into protoplasts of *S. erythraea* JC2 and stable thiostrepton resistant colonies are selected. Total DNA from one such colony is isolated and analysed by Southern hybridisation, 5 to confirm that the plasmid has integrated specifically into the chromosomal copy of the portion of the eryAIII gene that encodes the C-terminal thioesterase/cyclase. This strain is designated *S. erythraea* JC2/pAR33.

10 (ii) Production of a novel triketide lactone by *S. erythraea* JC2/pAR33

*S. erythraea* JC2/pAR33 is inoculated into sucrose-succinate medium containing 50  $\mu$ g/ml thiostrepton, 15 and allowed to grow for five days at 28-30°C. After this time, the broth is filtered, and extracted twice with an equal volume of ethyl acetate, and the combined ethyl acetate extracts are dried over anhydrous sodium sulphate and the ethyl acetate is removed by evaporation under 20 reduced pressure. Electrospray MS of the residue showed the presence of Ac-2-nor-3-epi-TKL (III, R=Mc) and 2-nor-3-epi-TKL (III, R=Et).



Example 20

### Construction of plasmid pAR8

Construction of a hybrid triketide lactone synthase  
containing the ery loading didomain and ery  
5 chain-terminating thioesterase/cyclase, and modules 11 and  
12 of the rap PKS

This example requires the initial construction of five  
separate plasmids, four housing separate elements of the  
10 target construct, and a fifth housing a gene conferring  
resistance to tetracycline. The inserts in these plasmids  
are sequentially combined by standard in vitro recombinant  
DNA techniques to form plasmid pAR5. A further three  
cloning steps lead to the final expression plasmid pAR8  
15 (Figure 12).

### Construction of plasmid pARLD

The segment of the ery AI gene from nucleotide 1 to  
20 nucleotide 1673, encoding the loading AT-ACP didomain, was  
amplified by PCR employing the CloneAmp procedure with the  
following two oligodeoxynucleotides as primers:

5' -ACGCGUACUAGUCCGATTAATTAAGGAGGACCATCAATGGCGGACCTGTCAAAGC  
25 TC-3' and  
5' -AUGGAGAUCCUCJCCGCTAGCGGTTCCGCGGGCGCCGCTTCGTTGGTCCGCGCGCG  
GGTTTCCC-3'

and plasmid pBK6.12 (Example 5) as template, to give plasmid pARLD.

#### Construction of plasmid pAR11

5

The segment of the rapC gene of *S. hygroscopicus* (Schwecke, T. et al. Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843) from nucleotide 112 to nucleotide 2095, the 5'- end of the DNA encoding rap module 11, is amplified by 10 PCR employing the CloneAmp procedure with the following two oligodeoxynucleotides as primers:

5'-AUGGAGAUCUCUCCGCTAGCGATTGTGGSTATGGCG-3'

and

15 5'-ACGCGUACUAGUCCATGCATCTGCAGCACGGCGGCCTCATCACCGGA-3'

and the DNA of recombinant bacteriophage  $\lambda$ -1C (Schwecke, T. et al., Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843) as the template. Approximately 30-60 ng of the PCR product (2.0 kbp) is digested with uracil DNA glycosylase 20 for 30 min at 37°C in the presence of 25 ng pAMP18 vector DNA, the mixture is cooled on ice and transformed into *E. coli* TG1 recO and individual colonies checked for their plasmid content. The desired plasmid (4.7 kbp) is identified by its restriction map and is designated pAR11.

25

#### Construction of plasmid pAR12

The segment of the rapC gene of *S. hygroscopicus*

(Schwecke, T. et al., Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843) from nucleotide 7405 to nucleotide 9396, the 3' end of the DNA encoding rap module 12, is amplified by PCR employing the CloneAmp procedure with the following two oligodeoxynucleotides as primers:

5'-ACGCGUACUAGUCCATGCATTCCCGAGCGGCGATCTGTGG-3'

and

5'-AUGGAGAUCUCUCCCGCGGCGCTGTACGCACCAGCTTC

AGCAGTGCCTC-3' and the DNA of recombinant bacteriophage 1-1C (Schwecke, T. et al., Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843) as template. Approximately 30-60 ng of the PCR product (2.0 kbp) is digested with uracil DNA glycosylase for 30 minutes at 37°C in the presence of 25 ng pAMP18 vector DNA, the mixture is cooled on ice and transformed into E. coli TG1recO and individual colonies are checked for their plasmid content. The desired plasmid (4.7 kbp) is identified by its restriction map and is designated pAR12.

20

#### Construction of pARTE

The 1.3 kbp segment of the eryAIII gene, extending by 132 nucleotides 3' of the eryAIII stop codon to a KpnI site, and encoding the C-terminal chain-terminating thioesterase/cyclase of DEBS, is amplified by PCR employing the CloneAmp procedure with the following two oligodeoxynucleotides as primers:



5'-ACGCGUACUAGUCCGCGGCCGCGATCCTCGGGCATTCCAGC-3'

and

5'-AUGGAGAUCUCUAAGCATTGGTAACTGTC-3', and plasmid pEXDB3

(Roberts, G. A. et al. Eur J. Biochem. (1993) 214:305-311)

5 as the template. Approximately 30-60 ng of the PCR product (1.3 kbp) is digested with uracil DNA glycosylase for 30 min at 37°C in the presence of 25 ng pAMP18 vector DNA, the mixture is cooled on ice and transformed into E. coli TG1 recO and individual colonies checked for their  
10 plasmid content. The desired plasmid (4.0 kbp) is identified by its restriction map and is designated pARTE.

#### Construction of plasmid pARTr

15 The 1.3 kbp segment of plasmid pBR322 containing the tetracycline resistance gene is amplified by the CloneAmp procedure with the following two oligodeoxynucleotides as primers:

20 5'-ACGCGUACUAGUATCTAGACCATGCATGTTTGACAGCTTATCATC-3'

and

5'-AUGGAGAUCUCUATCTAGACCATGCATGCCGCCGGCTTCCATTCA-3'

and plasmid pBR322 as the template. Approximately 30-60  
25 ng of the PCR product (1.3 kbp) is digested with uracil DNA glycosylase for 30 minutes at 37°C in the presence of 25 ng pAMP18 vector DNA, the mixture is cooled on ice and transformed into E. coli TG1recO and individual colonies

are checked for their plasmid content. The desired plasmid (4.0 kbp) is identified by its restriction map and is designated pARTr.

#### 5 Construction of plasmid pAR1

Plasmid pARLD is digested with NheI and HindIII, and ligated to the 2.0 kbp NheI-HindIII insert obtained from plasmid pAR11. The ligation mixture is transformed into  
10 E. coli TG1recO and individual colonies are checked for their plasmid content. The desired plasmid is identified by its restriction map and is designated pAR1.

#### Construction of plasmid pAR2

15

Plasmid pAR1 is linearised with NsiI and ligated with the NsiI fragment from pARTr. The ligation mixture is transformed into E. coli TG1recO and individual colonies are checked for their plasmid content. The desired  
20 plasmid is identified by its restriction map and is designated pAR2.

#### Construction of plasmid pAR3

25 Plasmid pAR2 is digested with SpeI and XbaI and the insert is ligated with plasmid pAR12 which has been linearised with SpeI. The ligation mixture is transformed into E. coli TG1recO and individual colonies are checked for their

plasmid content. The desired plasmid is identified by its restriction map and is designated pAR3.

#### Construction of plasmid pAR4

5

Plasmid pAR3 is digested with NsiI and the vector is ligated to the NsiI fragment of pARTr, containing the tetracycline resistance gene. The ligation mixture is transformed into *E. coli* TG1recO and individual colonies, 10 grown in the presence of tetracycline (12.5 µg/ml), are checked for their plasmid content. The desired plasmid is identified by its restriction map and is designated pAR4.

#### Construction of plasmid pAR5

15

Plasmid pAR4 is digested with NotI and XbaI and ligated with a NotI-XbaI fragment obtained by digestion of plasmid pARTE. The ligation mixture is transformed into *E. coli* TG1recO and individual colonies, grown in the presence of 20 tetracycline (12.5 µg/ml), are checked for their plasmid content. The desired plasmid is identified by its restriction map and is designated pAR5.

#### Construction of plasmid pAR5-2

25

A 7.2 kbp segment of the rapC gene of *S. hygroscopicus* is excised from cosmid 13 (Schwecke, T. et al., Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843) using BstXI and NdeI,

purified by gel electrophoresis, and ligated with plasmid pAR5 which has also been digested with BstXI and NdeI. The ligation mixture is transformed into *E. coli* TG1recO and individual colonies, grown in the presence of 5 tetracycline (12.5  $\mu\text{g/ml}$ ), are checked for their plasmid content. The desired plasmid (11.9 kbp) is identified by its restriction map and is designated pAR5-2.

#### Construction of plasmid pAR5-3

10

A 3.0 kbp segment of plasmid pAR5 is excised by digestion with NdeI, purified by gel electrophoresis, and ligated with plasmid pAR5-2 which had been linearised with NdeI. The ligation mixture is transformed into *E. coli* TG1recO 15 and individual colonies, grown in the presence of tetracycline (12.5  $\mu\text{g/ml}$ ), are checked for their plasmid content. The desired plasmid (14.9 kbp) is identified by its restriction map and is designated pAR5-3.

20

#### Construction of plasmid pAR8

A 12.2 kbp fragment of plasmid pAR5-3 is excised using PacI and XbaI, purified by gel electrophoresis, and 25 ligated with plasmid pRM52 (Example 4) which had been cut with PacI and XbaI. The ligation mixture is transformed into *E. coli* TG1recO and individual colonies, grown in the presence of tetracycline (30.3  $\mu\text{g/ml}$ ), are checked

for their plasmid content. The desired plasmid (14.9 kbp) is identified by its restriction map and is designated pAR8.

#### 5 Example 21

Construction of *S. erythraea* JC2/pAR8 and production of TKL derivatives

##### (i) Construction

10

Approximately 5-10 µg pAR8, isolated from *E. coli* DH10B (pAR8) is used to transform *S. erythraea* JC2 protoplasts and stable thiostrepton resistant colonies are selected. One of these colonies is selected and total DNA is

15 prepared for Southern hybridisation analysis, to confirm that the plasmid has integrated specifically into the chromosomal copy of the portion of the *eryAIII* gene that encodes the C-terminal thioesterase/cyclase. This strain is designated *S. erythraea* JC2/pAR8

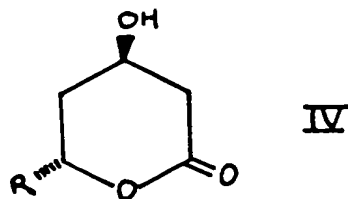
20

##### (ii) Production of 2,4-bisnor-3-epi-TKL and (Ac)-2,4-bisnor-3-epi-TKL

A colony of *S. erythraea* JC2/pAR8 is picked and  
25 transferred to sucrose-succinate medium supplemented with 50 µg/ml thiostrepton and allowed to grow at 30°C. After 3 days the broth is filtered and extracted twice with an equal volume of ethyl acetate. The combined ethyl acetate

extracts are dried over anhydrous sodium sulphate and concentrated under reduced pressure. GC-MS of the residue shows the presence of 2,4-bisnor-3-epi-TKL (IV, R=E+) and (Ac)-2,4-bisnor-3-epi-TKL (IV, R=Me)

5



10

#### Example 22

#### Construction of plasmid pE1A2TE

Plasmid pE1A2TE (like plasmid pE1A2TE-2 also described 15 herein) consists of a pT7.7 derived plasmid containing a hybrid Type I PKS gene comprising the ery loading module, the first extension module of the ery PKS, then the second extension module of the avr PKS, and the thioesterase of the ery PKS. It is constructed via several intermediate 20 plasmids as follows (Figure 13).

#### Construction of plasmid pIG70

Plasmid pVE1446 which contains a portion of the avermectin 25 PKS genes was obtained from E. coli ATCC 68250. Plasmid pVE1446 was digested with BamHI and the 7.0 kbp fragment between coordinates 6 05 and 13.05 was purified by gel electrophoresis and ligated into plasmid pUC119 which had

been linearised with BamHI. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. Of the two possible orientations of the BamHI insert pIG70 was selected such that when digested with PstI fragments of approximately 2.0 and 8.6 kbp were obtained and when digested with EcoRI fragments of approximately 5.1 and 5.5 kbp were obtained.

#### 10 Construction of plasmid pIG71

Plasmid pVE1446 which contains a portion of the avermectin PKS genes was obtained from E. coli ATCC 68250. Plasmid pVE1446 was digested with BamHI and the 7.1 kbp fragment between coordinates 13.05 and 20.15 was purified by gel electrophoresis and ligated into plasmid pUC119 which had been linearised with BamHI. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. Of the two possible orientations of the BamHI insert pIG71 was selected such that when digested with EcoRI and XhoI 2 fragments of approximately 5 kbp were obtained.

#### 25 Construction of plasmid pIG70ΔPst

pIG70 was cut with SacI and religated. pIG70ΔPst was isolated after transformation into E. coli TG1 recO.

### Construction of plasmid pIG70ΔEco

pIG70 was cut with EcoRI and religated. pIG70ΔEco was  
5 isolated after transformation into E. coli TG1 recO

### Construction of plasmid pIG71ΔSac

10 pIG71 was cut with SacI and religated. pIG71ΔSac was  
isolated after transformation into E. coli TG1 recO

### Construction of plasmid pIGPCRstart

15

50 pmol of each of synthetic oligonucleotides 8985  
(5'-GAGCAGTCGTTCCGAGATCTCGGCTTCGATTCA-3') which introduced  
a BglII site and 9204 (5'-GGGAGGAGATCAGATCCCAGAAGT-3')  
were used by PCR to amplify a 300 bp product from 60 ng

20 pIG70ΔEco. The PCR product was end-repaired,

phosphorylated and ligated into pUC18 that had been  
linearised with SmaI and dephosphorylated. The ligation  
mixture was used to transform E. coli TG1 recO and  
individual colonies were checked for their plasmid

25 content. The orientation of pIGPCRstart was identified by  
a double restriction enzyme digest with EcoRI and BglII to  
give a pattern that included a 300 bp fragment.



## Construction of plasmid pIGPCrend

50 pmol of each of synthetic oligonucleotides 8986  
(5'-GAGGGAGTCGAACCGAGATCTCGGAACGCGCGG-3') which introduced  
5 a BglIII site and 9205  
(5'-GGGGGATCCTGGGGTCGGCCGGGCAGGGCAA-3') were used by PCR  
to amplify a 440 bp product from 60 ng pIG71ΔSac. The PCR  
product was end-repaired, phosphorylated and ligated into  
pUC18 that had been linearised with SmaI and  
10 dephosphorylated. The ligation mixture was used to  
transform E. coli TG1 recO and individual colonies were  
checked for their plasmid content. The orientation of  
pIGPCrend was identified by its restriction enzyme digest  
pattern.

15

## Construction of plasmid pIGstart+middle

Plasmid pIGPCRstart was digested with PstI and the 300 bp  
20 fragment was purified by gel electrophoresis and ligated  
into plasmid pIG70ΔPst which had been linearised with PstI  
and dephosphorylated. The ligation mixture was used to  
transform E. coli TG1 recO and individual colonies were  
checked for their plasmid content. Plasmids which  
25 contained the correct orientation of the PstI-PstI insert  
were identified by DNA sequencing.

### Construction of plasmid pIGave2Bgl

Plasmid pIGstart+middle was digested with BamHI and the 5.0 kbp fragment was purified by gel electrophoresis and ligated into plasmid pIGPCRend which had been cut with BamHI and dephosphorylated. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. Plasmids which contained the correct orientation of the BamHI-BamHI insert were identified by DNA sequencing.

### Construction of plasmid pE1A2TE

Plasmid pIGave2Bgl was digested with BglII and the 6 kbp fragment was purified by gel electrophoresis and ligated into plasmid pKW15 (Example 16) which had been linearised with BglII and dephosphorylated. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. Plasmids which contained the correct orientation of the BglII-BglII insert were identified by restriction enzyme digest with EcoRI.

25

### Example 23

#### Construction and use of plasmid pIG2

## (i) Construction

Plasmid pE1A2TE was digested with NdeI and XbaI and the 11 kbp fragment was purified by gel electrophoresis and ligated into plasmid pRM52 (Example 4) which had been cut with NdeI and XbaI. The ligation mixture was used to transform *E. coli* TG1 recO and individual colonies were checked for their plasmid content.

10

(ii) Construction of *S. coelicolor* CH999/pIG2

Plasmid pIG2 which had been isolated from *E. coli* ET12567 (MacNeil, D. J. et al. Gene (1992) 111:61-68) was transformed into protoplasts of *S. coelicolor* CH999 and stable thiostrepton resistant colonies were isolated. Individual colonies were checked for their plasmid content and the presence of plasmid pIG2 was confirmed by its restriction pattern.

20

## Example 24

## Construction of plasmid pIG102

25 Plasmid pE1A2TE was digested with NdeI and XbaI and the 11 kbp fragment was purified by gel electrophoresis and ligated into plasmid pCJR101 (Example 2) which had been cut with NdeI and XbaI. The ligation mixture was used to

transform *E. coli* TG1 *recO* and individual colonies were checked for their plasmid content.

5

#### Example 25

#### Construction of plasmid pMO7

10 Plasmid pMO7 (like plasmid pMO107 also herein described) is an SCP2\*-based plasmid containing a PKS gene comprising the ery loading module, the first and second extension modules of the ery PKS and the ery chain-terminating thioesterase, except that the DNA segment encoding the  
15 methylmalonyl-CoA:ACP acyltransferase within the first ery extension module has been specifically substituted by the DNA encoding the malonyl-CoA:ACP acyltransferase of module 13 of the rap PKS. It was constructed via several intermediate plasmids as follows (Figure 14).

20

#### Construction of plasmid pMO1

The approximately 1.3 kbp DNA segment of the *eryAI* gene of *S. erythraea* extending from nucleotide 1948 to nucleotide  
25 3273 of *eryAI* (Donadio, S. et al. Science (1991) 252:675-679) was amplified by PCR employing as primers the synthetic oligonucleotides:  
5'-CATGCTCGAGCTCTCCTGGGAAGT-3' and

5'-CAACCCTGGCCAGGGAAGACGAAGACGG-3', and plasmid pNTEP2 (Example 5) as template. The PCR product was end-repaired and ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline 5 phosphatase. The ligation mixture was used to transform *E. coli* TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO1 (3.9 kbp), in which the StuI site bordering the insert is adjacent to the HindIII site in the polylinker, was 10 identified by its restriction pattern.

#### Construction of plasmid pMO2

The approximately 0.85 kbp DNA segment of the *rapA* gene of 15 *S. hygroscopicus*, extending from nucleotide 1643 to nucleotide 2486 of *rapA*, was amplified by PCR employing as primers the following oligonucleotides:

5'-TTCCCTGGCCAGGGGTCGCAGCGTG-3' and 20 5'-CACCTAGGACCGCGGACCACTCGAC-3', and the DNA from the recombinant bacteriophage  $\lambda$ -1E (Schwecke, T. et al. Proc. Natl. Acad. Sci. USA (1995) 92:7839-7843) as the template. The PCR product was end-repaired and ligated with plasmid pUC18, which had been linearised by digestion with SmaI 25 and then treated with alkaline phosphatase. The ligation mixture was used to transform *E. coli* TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO2 (3.5 kbp) was

identified by its restriction pattern.

#### Construction of plasmid pMO3

5 The approximately 1.7 kbp DNA segment of the eryAI gene of *S. erythraea* extending from nucleotide 4128 to nucleotide 5928 of eryAI, was amplified by PCR employing as primers the synthetic oligonucleotides:

5'-TGGCCAGGGAGTCGGTGCACCTAGGCA-3' and

10 5'-GCCGACAGCGAGTCGACGCCGAGTT-3' and plasmid pNTEP2 as template. The PCR product was end-repaired and ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform *E. coli* TG1 recO  
15 and individual colonies were checked for their plasmid content. The desired plasmid pMO3 (4.4 kbp), in which the BalI and AvrII sites are adjacent to the HindIII site of the polylinker, was identified by its restriction pattern.

20

#### Construction of plasmid pMO4

Plasmid pMO1 was digested with HindIII and BalI and the 1.3 kbp insert was ligated with plasmid pMO3 which had  
25 been digested with HindIII and BalI. The ligation mixture was used to transform *E. coli* TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO4 (5.6 kbp) was identified by its

restriction pattern.

#### Construction of plasmid pMO5

5 Plasmid pMO4 was digested with *Stu*I and the 3.0 kbp insert was ligated with plasmid pNTEP2 which had been digested with *Stu*I and the vector purified by gel electrophoresis to remove the 3.8 kbp insert. The ligation mixture was transformed into *E. coli* TG1 *recO* and individual colonies  
10 were checked for their plasmid content. The desired plasmid pMO5 (12.8 kbp) was identified by its restriction pattern.

#### Construction of plasmid pMO6

15

Plasmid pMO2 was digested with *Bal*I and *Avr*II and the insert was ligated with plasmid pMO5 which had been digested with *Bal*I and *Avr*II. The ligation mixture was used to transform *E. coli* TG1 *recO* and individual colonies  
20 were checked for their plasmid content. The desired plasmid pMO6 (13.5 kbp) was identified by its restriction pattern.

#### Construction of plasmid pMO7

25

Plasmid pMO6 was digested with *Nde*I and *Xba*I and the insert was ligated with plasmid pRM52 (Example 4) which had been digested with *Nde*I and *Xba*I and purified by gel

electrophoresis. The ligation mixture was transformed into *E. coli* TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO7 (also designated pRMAT2) was identified by its restriction 5 pattern.

#### Example 26

Construction of *S. coelicolor* CH999/pMO7 and production of TKL derivatives

10

##### (i) Construction

Plasmid pMO7 which had been isolated from *E. coli* ET12567 (MacNeil, D. J. et al. *Gene* (1992) 111:61-68) was transformed into protoplasts of *S. coelicolor* CH999 and 15 stable thiostrepton resistant colonies were isolated. Individual colonies were checked for their plasmid content and the presence of plasmid pMO7 was confirmed by its restriction pattern.

20 (ii) Production and isolation of 4-nor-TKL and (Ac)4-nor-TKL using *S. coelicolor* CH999/pMO7

*S. coelicolor* CH999/pMO7 was inoculated into YEME medium containing 50 µg/ml thiostrepton and allowed to grow for 25 five days at 28-30°C. After this time the broth was filtered to remove mycelia and the pH adjusted to pH 3. The broth was extracted twice with two volumes of ethyl acetate and the combined ethyl acetate extracts were



washed with an equal volume of saturated sodium chloride, dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure, to give about 200 mg crude product. This was digested with 2 ml of 5 methanol, and mixed with 0.5 g of dry silica gel, and then subjected to flash chromatography on a column of the same material (1 cm x 15 cm) The column was eluted with diethyl ether, and fractions of 10 ml each were collected. Fractions 4-8 were pooled, and the diethyl ether was 10 evaporated to leave about 10 mg of oily residue containing the compounds of interest. These were purified further by hplc on an octadecylsilica reverse phase column (10 mm x 25 cm) eluted at a flow rate of 2 ml/minute first with an isocratic mixture of water/methanol 75:25 (vol/vol) for 15 five minutes, then with a linear gradient of increasing methanol, reaching water/methanol 55/45 (vol/vol) after 30 minutes. After about 11 minutes, fractions were collected containing, as the minor component, (Ac)4-nor-TKL (II, R<sub>1</sub>=Me, R<sub>2</sub>=H, R<sub>3</sub>=Me) and after about 18 minutes 20 fractions were collected containing, as the major component, 4-nor-TKL (II, R<sub>1</sub>=Me, R<sub>2</sub>=H, R<sub>3</sub>=ET).

The <sup>1</sup>H spectrum of 4-nor-TKL was determined using a Bruker AM-400 NMR spectrometer. Found: δH (400 MHz, CDCl<sub>3</sub>) 4.18 25 (1H, dtd, 11.8, 6.1, 2.9 Hz, H-5), 3.75 (1H, ddd, 11.0, 10.0, 4.0 Hz, H-3), 2.35 (1H, dq, 10.0, 7.0 Hz, H-2), 2.20 (1H, ddd, 13.3, 4.0, 2.9 Hz, H-4eq), 1.6 - 1.88 (3H, m, 2xH-6, H-4ax), 1.41 (1H, d, 7.0 Hz, CH<sub>3</sub>-3'), 1.01 (1H, t,

7.5 Hz, CH<sub>3</sub>-7) ppm.

The <sup>13</sup>C NMR spectrum of 4-nor-TKL was also determined (100 MHz, CDCl<sub>3</sub>): 173.3 (C-1), 77.7 (C-5), 70.4 (C-3), 45.1 (C-2), 37.7 (C-4), 28.8 (C-6), 13.5 (C-3'), 9.1 (C-7).

#### Example 27

Construction of plasmid pMO107 and production of TKL  
10 derivatives

##### (i) Construction

Plasmid pMO6 was digested with NdeI and XbaI and the  
15 insert was ligated with plasmid pCJR101 (Example 2) which  
had been digested with NdeI and XbaI and purified by gel  
electrophoresis. The ligation mixture was transformed  
into E. coli TG1 recO and individual colonies were checked  
for their plasmid content. The desired plasmid pMO107 was  
20 identified by its restriction pattern.

(ii) Production and isolation of 4-nor-TKL and  
(Ac)4-nor-TKL using S. erythraea JC2/pMO107

25 S. erythraea JC2/pMO107 was prepared by standard  
techniques (c.f. Example 26(i)) and inoculated into  
sucrose-succinate medium containing 50 µg/ml thiostrepton  
and allowed to grow for three-five days at 28-30°C. After

this time the broth was filtered to remove mycelia and the pH adjusted to pH 3. The broth was extracted three times with quarter volumes of ethyl acetate and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure, to give about 10 mg/L crude product. This was digested with 2 ml of methanol, and mixed with 0.5 g of dry silica gel, and then subjected to flash chromatography on a column of the same material (1 cm x 15 cm). The column was eluted with diethyl ether, and fractions of 10 ml each were collected. Fractions 4-8 were pooled, and the diethyl ether was evaporated to leave about 15 mg of oily residue containing the compounds of interest. These were purified further by hplc on an octadecylsilica reverse phase column (10-mm x 25 cm) eluted at a flow rate of 2 ml/minute first with an isocratic mixture of water/methanol 75:25 (vol/vol) for five minutes, then with a linear gradient of increasing methanol, reaching water/methanol 55/45 (vol/vol) after 30 minutes. After about 11 minutes, fractions were collected containing, as the minor component, (Ac)4-nor-TKL and after about 18 minutes fractions were collected containing, as the major component, 4-nor-TKL.

25 The  $^1\text{H}$  and  $^{13}\text{C}$  spectra of the purified 4-nor-TKL and (Ac)4-nor-TKL were identical with the spectra obtained for authentic material.

## Example 28

Construction of *S. erythraea* ERMD1, carrying a hybrid PKS gene in which the avr loading didomain is substituted for the ery loading didomain of *S. erythraea* NRRL 2338

5

## (i) Construction of plasmid pAVLD

Plasmid pCRabc (Example 9) was linearised with BamHI and ligated to pIJ702 previously digested with BglII. The mixture contained the desired plasmid pAVLD (Figure 15). The ligation mixture was transformed into *E. coli* TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pAVLD was identified by its restriction pattern (Figure 15).

15

(ii) Construction of *S. erythraea* ERM D1

Approximately 5-10 mg of pAVLD, isolated from *E. coli* TG1recO(pAVLD) was transformed into *S. erythraea* NRRL2338 and stable thiostrepton resistant colonies were isolated. One of these colonies was selected and total DNA was digested with PstI and analysed by Southern hybridisation employing as a probe the insert from plasmid pCRc which contains the fragment of the ery AI gene encoding the ketosynthase domain KS1. The analysis showed positively-hybridizing PstI fragments of 8.5 kbp, 4.8 kbp and 33 kbp, indicating the presence of two tandemly integrated copies of pAVLD (Figure 16).

25

## Example 29

## Isolation of erythromycins altered at C-13

A 50 ml fermentation of *S. erythraea* ERMD1 was carried out on tap water medium and after 4 days at 30°C the mycelium was harvested and used to inoculate 1.5 litres of sucrose-succinate medium containing thiostrepton (50µg/ml).

10 After growth at 30°C for 4 days, the whole broth was extracted twice with an equal volume of ethyl acetate.

The combined extracts were concentrated under reduced pressure and subjected twice to preparative thin layer chromatography on silica plates (20 x 20cm) eluted with chloroform/methanol/.88 ammonia 8:2:0.01 (by vol). The products were separated by hplc on a PhaseSep C18 base-deactivated reverse phase column S5odS (octadecylsilica) 6 (4.6mm x 250mm), eluted with methanol/0.5% ammonium acetate (70:30 (vol/vol), at 1 ml.min. Fractions were collected between 7 and 11 minutes from three separate injections, and the pooled fractions were re-injected in ten separate injections. The order of elution from the column was: erythromycin B analogues, followed by erythromycin D analogues and erythromycin A analogues. B and D analogues emerged after 8-10 minutes, erythromycin A analogue 3-4 minutes later. The analogues containing a C-4 (isobutyryl) starter unit are eluted earlier, with the

analogues with C-5 (isovaleryl) starter unit emerging several minutes later, although the C-4 late (eryA analogue) and the early C-5 (erythromycins B and D analogue) overlap. High resolution MS gave results for C-5 4 eryA, eryB and eryD analogues, and for C-5 eryA and eryB analogues, which correspond closely to those calculated:

Analogue	Calc'd Mass	Measured Mass
C5-eryA	762.5004	762.5021
10 C4-eryA	748.4847	748.4820
C5-eryB	746.4898	748.5077
C4-eryB	732.4898	732.4933

In these experiments natural erythromycins were present only in low or undetectable amounts, and there were no detectable amounts of eryC analogues. The overall concentration ratio of C-4/C-5 compounds in the fermentation broth, as assessed by ESMS of ethyl acetate extracts of broths, was between 4:1 and 6:1 in favour of C-4 compounds. The ratio of A:B:D analogues is variable, about 15:60:25, but with an increasing proportion of A analogues as the fermentation proceeds. The total yield of erythromycins is about 400 µg/litre.

25

#### Example 30

Construction and use of *S. erythraea* NRRL2338/pRMTE

(i) Construction

Approximately 5 µg of plasmid pRMTE (Example 6) isolated from *E. coli* TGI recO (pRMTE) was transformed into 5 protoplasts of *S. erythraea* NRRL2338 and stable thiostrepton resistant colonies were isolated. One of these was selected and designated *S. erythraea* NRRL2338/pRMTE.

10

(ii) Enhanced production of erythromycin A and erythronolides using *S. erythraea* NRRL2338/pRMTE

*S. erythraea* NRRL2338/pRMTE was grown in sucrose and 15 succinate medium containing 50 µg/ml thiostrepton at 28-30°C. After 3 days the whole broth was extracted twice with an equal volume of ethyl acetate, the combined ethyl acetate extracts were washed with saturated sodium chloride solution, dried over anhydrous sodium sulphate, 20 and concentrated under reduced pressure.

Examination of the extract by thin layer chromatography on silica plates eluted with isopropyl ether:methanol:ammonium hydroxide 75:35:1 (by volume) showed the presence of several components. Electrospray 25 mass spectrometry of the extracts revealed the presence of a mixture of erythromycin A, erythronolide B (EB) and 6-deoxyerythronolide B (6-DEB), together with minor amounts of (Ac)-6-DEB as its sodium adduct, (Ac)-EB as its

Na adduct (411.1), and EB as its Na adduct (424.1), and also TKL (m/e 159.1). The yield of erythromycin A plus erythronolide B was about 500 mg/L of medium, compared to about 50 mg /L produced by *S. erythraea* NRRL2338 fermented under identical conditions. Cells of *S. erythraea* NRRL2338/pRMTE harvested from the fermentation broth after 3 days were disrupted and their protein content was examined by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. Three high molecular weight bands, corresponding to the erythromycin PKS multienzyme subunits DEBS1, DEBS2 and DEBS3 were observed, approximately ten times more intense than the same protein band seen from cell extracts of *S. erythraea* NRRL2338 prepared by the same procedure (Caffrey, P. et al. FEBS Letters (1992) 304:225-228).

An identical fermentation of *S. erythraea* NRRL2338/pRMTE was carried out except that the medium was supplemented with 5 mM potassium propionate. After three days the broth was extracted with ethyl acetate as before, and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate, and concentrated. Preparative TLC using the system isopropyl ether:methanol:ammonium hydroxide 75:35:1 (by volume) separated two major components. Analytical TLC showed that the faster running component (Rf 0.8) has the same mobility as authentic 6-DEB; and the slower migrating material was an approximately equal mixture of a component of Rf 0.63, co-migrating with an



authentic sample of TKL; and a component of Rf 0.60, with the same mobility as an authentic sample of EB.

Electrospray mass spectrometry (ESMS) on a VG BioQ mass spectrometer operated in positive ion mode showed that the component of Rf 0.75 had  $m/e$  387.4, as required for 6-DEB. ESMS of the mixture of the components with Rf values 0.60 and 0.63 confirmed the presence of TKL and EB.

#### 10 Example 31

Construction and use of *S. erythraea* TER43/pRMTE

##### (i) Construction

Approximately 5 mg of plasmid pRMTE was transformed into protoplasts of *S. erythraea* TER43 (Cortes, J. et al., Science (1995) 268:1487-1489) and stable thiostrepton resistant colonies were isolated. One of these was selected and designated *S. erythraea* TER43/pRMTE.

##### 20 (ii) Enhanced production of TKL using *S. erythraea* TER43/pRMTE

*S. erythraea* TER43/pRMTE was inoculated into 1L sucrose-succinate medium and allowed to grow for 3 days at 25 28-30°C. After 3 days, the broth was extracted twice with an equal volume of ethyl acetate, and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate and concentrated. Analysis of the extract by electrospray

mass spectrometry (operated in the positive ion mode) showed the presence of TKL (m/e 173.1) and of (Ac)-TKL (m/e 159.1). The combined yield of triketide lactones was 100 mg/L, compared with 10 mg/L obtained by fermentation of *S. erythraea* TER43 under identical conditions. Cells of *S. erythraea* TER43/pRMTE, harvested from the fermentation broth after 3 days, were disrupted and their protein content was examined by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. A high molecular weight band, corresponding to the erythromycin PKS subunit DEBS1 with the attached thioesterase domain (Cortes, J. et al. Science (1995) 268:1487-1489) was observed, approximately ten times more intense than the same protein band seen from cell extracts of *S. erythraea* prepared by the same procedure.

#### Example 32

Construction and use of *S. erythraea* NRRL2338/pCJRTE

20

##### (i) Construction

Approximately 5 µg of plasmid pCJRTE is transformed into protoplasts of *S. erythraea* NRRL2338 and stable thiostrepton resistant colonies are isolated. From several such colonies, total DNA is obtained and analysed by Southern hybridisation, to confirm that the plasmid has integrated specifically into the *eryA* genes by homologous recombination, so as to place the resident *eryA* genes

under the control of the actI promoter derived from plasmid pCJRTE, while the DEBS1-TE gene borne by the incoming plasmid is placed by the integration event under the control of the chromosomal eryA promoter.

5

(ii) Enhanced production of erythromycins and their precursors using *S. erythraea* NRRL2338/pCJRTE.

*S. erythraea* NRRL2338/pCJRTE is inoculated into  
10 sucrose-succinate medium containing 50 mg/ml thiostrepton and allowed to grow for four days at 30°C. After this time the broth is filtered to remove mycelia and then extracted twice with an equal volume of ethyl acetate. The combined ethyl acetate extracts are analysed by mass  
15 spectrometry and it is found that the mixture contains erythromycin A, accompanied by 6-DEB, (Ac)-DEB, TKL and (Ac)-TKL, in total amounts 100 mg/L, or 5 times the total amount of erythromycins and precursors of erythromycins that are obtained using *S. erythraea* NRRL2338 under the  
20 same conditions.

#### Example 33

Construction and use of *S. erythraea* JC2/pCJRTE

25

(ii) Construction

Approximately 5 µg of plasmid pCJRTE is transformed into protoplasts of *S. erythraea* JC2 and stable thiostrepton

resistant colonies are isolated. From several such colonies, total DNA is obtained and analysed by Southern hybridisation, to confirm that the plasmid has integrated specifically into the portion of the *eryAIII* gene that encodes the C-terminal thioesterase/cyclase, by homologous recombination.

(ii) Enhanced production of triketide lactones using *S. erythraea* JC2/pCJRTE

*S. erythraea* JC2/pCJRTE is inoculated into sucrose-succinate medium containing 50 µg/ml thiostrepton and allowed to grow for four days at 30°C. After this time the broth is filtered to remove mycelia and then extracted twice with an equal volume of ethyl acetate. The combined ethyl acetate extracts are analysed by mass spectrometry and NMR and it is found that the major product is TKL, and the minor product (Ac)TKL, in total yields (100 mg/L) 10 fold greater than obtained using *S. erythraea* TER43.

Example 34

25 Construction and use of *S. erythraea* NRRL2338/pIG1

(i) Construction

Approximately 5 µg of plasmid pIG1 is transformed into

protoplasts of *S. erythraea* NRRL2338 and stable thiostrepton resistant colonies are isolated. From several such colonies, total DNA is obtained and analysed by Southern hybridisation, to confirm that the plasmid has integrated specifically into the portion of the *eryAIII* gene that encodes the C-terminal thioesterase/cyclase, by homologous recombination.

(ii) Production of 14-membered lactones using *S. erythraea* NRRL/pIG1

*S. erythraea* NRRL/pIG1 is inoculated into tap water medium containing 50 µg/ml thiostrepton and allowed to grow for four days at 30°C. After this 20 ml of the mycelium is used to seed 500 ml of sucrose-succinate medium containing 50 mg/ml thiostrepton, in a 2L flask with a single spring to reduce clumping, shaken at 280 rpm. After between 3.5 and 6 days, the broth is filtered to remove mycelia and then extracted three times with a quarter volume of ethyl acetate. The combined ethyl acetate extracts are dried over anhydrous sodium sulphate and solvent removed by evaporation. Analysis of the product mixture using GC and electrospray MS revealed that of a total of 5-6 mg/L of 14-membered macrolide products, the major component was (s-pent)-erythromycin D (about 1.5 mg/L), with other components present being (s-pent)-erythromycin B and (s-pent)-erythromycin A; (i-but)-erythromycins A, B and D; and small amounts of

natural erythromycins A, B and D. The extracts also contained significant amounts (11 mg/l) of TKL's: (s-pent)-TKL (5mg/l), (i-but)-TKL and TKL. (NB s-pent and i-but indicate 1-methylpropyl and isopropyl side-chains, respectively, corresponding to the use of s-pentanoyl and i-butanoyl starter substrates.)

#### Example 35

#### 10 Determination of antibiotic activity of novel erythromycin A analogues

A 3 ml overnight culture of *Bacillus subtilis* ATCC 6633 was grown at 30°C in nutrient broth (Difco). 200ml of nutrient 1.5% agar (difco) at 46°C was seeded with 1ml of the *B. subtilis* culture and poured immediately into petri dishes (25 ml/plate). After drying the plates in a laminar flow hood for 15 minutes, wells (0.4mm in diameter) were cut using a cork borer and 20 microlitres of the test compound as a solution in ethanol (5-10 mg/L) was added to each well. The plates were kept at 4°C for 5-7 hours to allow the compound to diffuse, and the plates were then incubated overnight at 30°C. Clear zones of growth inhibition were seen with both (i-but)- and (s-pent)- erythromycin A.

Although the present invention is illustrated by the

examples listed above, they should not be regarded as limiting the scope of the invention. The above descriptions illustrate first, how a specific promoter for a Type II PKS gene set, coupled to its specific cognate activator gene, contrary to expectation, may be used to achieve controlled and enhanced expression of Type 1 PKS genes in a heterologous host. Examples of these hosts that are given are *S. erythraea* and *S. avermitilis*, but it will be evident to those skilled in the art that alternative hosts, drawn from a wide range of actinomycetes, will equally well serve as expression hosts. Similarly, although the actI promoter and its cognate activator gene actII-orf4 have been used in these Examples, it will be evident to those skilled in the art that other Type II PKS promoter/activator gene combinations are well-known and characterised which will be equally efficacious in directing the controlled and enhanced expression of Type 1 PKS genes in heterologous cells drawn from a wide range of actinomycetes. Examples of such promoter/activator gene combinations include the promoters of the dnr gene cluster and the dnrI activator gene from the daunorubicin gene cluster of *Streptomyces peucetius*: (Madduri, K. and Hutchinson, C.R. J. Bacteriol (1995) 177:1208-1215) and the promoter of the gene redX and the activator gene redD from the undecylprodigiosin gene cluster of *S. coelicolor* (Takano, E. et al. Mol. Microbiol. (1992) 2: 2797-2804).

Secondly, the above descriptions illustrate for the first time the construction of hybrid Type I PKS genes and their use to obtain novel polyketide products of utility as chiral synthetic intermediates or as bioactive materials such as antibiotics. Hybrid PKS genes have been constructed either by substitution of loading modules, or by substitution of individual domains in extension modules; or by substitution of whole modules. Thus, the replacement of the ery loading module by the avr loading loading module has been described herein to obtain either novel erythromycin A analogues or triketide lactones. It will readily occur to those skilled in the art that other alterations of the ery loading module can be obtained through its replacement with the loading module of other Type I PKS gene sets. Examples of such alterations include replacement with the loading module of the rap PKS; and with the loading module of the FK506-producing PKS. Such alterations will lead to the synthesis of polyketides specifically altered in their starter unit.

It is well-known to those skilled in the art that the avr loading module is capable of accepting a wide range of non-natural carboxylic acids as alternative starter units, when these are included in the fermentation medium.

Therefore in the light of the present invention, it is evident that in addition to the synthesis of novel erythromycin A derivatives in which the C-13 substituent is isopropyl or sec-butyl instead of ethyl, which has been



shown here, many other novel erythromycin A derivatives can be obtained by feeding of the appropriate non-natural carboxylic acids (or compounds convertible to them by fermentation) to an appropriate strain housing the hybrid  
5 PKS, such non-natural carboxylic acids having in general the formula  $R\text{-COOH}$ , where R is an alpha-branched group, and where the carbon bearing the  $\text{-COOH}$  group is also attached to at least two other atoms or groups other than hydrogen, with the preferred non-natural carboxylic acids  
10 being those described for the production of non-natural avermectins in European Patent EP 214,731, March 18 1987, Pfizer). The resulting novel analogues of erythromycin A can be converted, by procedures well understood in the art, into further novel semi-synthetic derivatives of  
15 erythromycin A, of considerable utility in the treatment of bacterial infection, including for example ketolides and azalides. These embodiments of the invention are novel chiral materials of potential utility in the chemical synthesis of valuable bioactive products. The  
20 products which are 14-membered macrolides are novel erythromycin A analogues which are highly valuable antibacterial agents having the same microbial targets as do the known erythromycins and the semi-synthetic derivatives of known erythromycins, such as the ketolides  
25 disclosed in French patents Nos. 2697523 (06/05/94) Roussel Uclaf; 269724 (06/05/94) Roussel Uclaf; and 2702480 (16/09/94) Roussel Uclaf.

It will be evident to those skilled in the art that the replacement of the ery loading module by the loading module of the rap PKS will also lead to novel and useful analogues of erythromycin A, in which the natural  
5 propionate starter unit is substituted by a cycloalkylcarboxylic acid starter unit. Further examples of the formation of such hybrid Type I PKS include, but are not limited to, the replacement of the rap loading module in *Streptomyces hygroscopicus* by the avr loading  
10 module, leading to the formation of non-natural rapamycins; and the replacement of the avr loading module in *Streptomyces avermitilis* by the rap loading module, leading to the formation of further examples of non-natural avermectins. The present invention also  
15 encompasses mutants in which more than one of the genetic manipulations described in the examples are combined.

In the light of the present invention, it will also be evident that alterations in the specificity of the loading  
20 module of a Type I PKS can alternatively be achieved by the mutation of the genes encoding the natural loading module, and then selection for the desired altered specificity, as practised for example in the technique of in vitro gene shuffling (Stemmer, W. P. Nature (1994)  
25 370:389-391).

The examples listed above also teach the construction and use of a low copy number plasmid vector pCJR101 as a

vector for delivery of PKS genes into suitable actinomycete hosts. Plasmid pCJR101 is derived from the plasmid SCP2\* (Bibb, M. J. and Hopwood, D. A. J. Gen. Microbiol. (1977) 154:155-166) found in the strain

5 *Streptomyces coelicolor* M110 deposited for example at the Northern Regional Research Laboratory, Peoria, Illinois, USA under the accession number NRRL 15041. Plasmid SCP2\* has been previously used in the construction of several useful vectors such as pIJ2839 (Ingram, C. et al. J.

10 *Bacteriol.* (1989) 171:6617-6624); plasmid pHJL197 (Larson, J. L. and Hershberger, C. L. J. *Bacteriol.* (1983) 157:314-317) and pRM5 (McDaniel, R. et al. *Science* (1993) 262:1546-1550). It will be evident to those skilled in the art that either these or other SCP2\*-based plasmids

15 may be substituted for pCJR101 either directly or after modification of the vector to introduce suitable promoter linked to the PKS genes, as demonstrated by the use of plasmid pRM5 in several Examples described herein. High copy number vectors such as plasmid pGM8 (Muth, G. et al.

20 *Mol. Gen. Genet.* (1989) 219:341-350) derived from the *Streptomyces ghanaensis* plasmid pGS5 are also suitable as substitutes for pCJR101, as are integrative vectors such as plasmid pSAM2 (Murakami, T. et al. J. *Bacteriol.* (1989) 171:1459-1477). Those skilled in the art will readily

25 appreciate the versatility of approaches to increasing the rate of biosynthesis of natural or non-natural complex polyketides such as macrolides and polyethers through heterologous use of type II PKS activator genes and their

cognate promoters as disclosed here, in numerous derivatives of vectors well known in the art as useful for genetic engineering in actinomycetes.

5 In the construction of hybrid type I PKS genes, the Examples teach how the structural genes encoding both donor and acceptor PKS components may be spliced together to create functional catalysts capable of bringing about the synthesis of novel polyketides. The present invention  
10 shows that in choosing where the junction will be made between the donor and the acceptor DNA it is, surprisingly, not necessary to limit the choice to positions known or predicted to lie between domains in so-called linker regions. Instead it is preferred for  
15 junctions to be in the edge regions of domains (particularly KS or AT domains), where the sequences are highly conserved. Further, creation of junctions that lead to conservative changes in amino acid sequence at such junctions in the gene product are tolerated. It is  
20 also evident that for the purposes of creating a hybrid, PKS modules may be combined from two or more natural PKS. In the examples given here, donor DNA is spliced into the acceptor DNA at a position variously in the acyl carrier protein (ACP) domain or in the ketosynthase (KS) domain of  
25 a module, but the scope of the invention includes hybrid PKS where the junctions between homologous domains are chosen to lie within any of the constituent parts of a type I PKS module. However, it will be found most

advantageous to select a position for each junction that lies within a domain, and close to one edge, so that the specificity of the chimaeric module is readily predictable, and so that disturbance of its proper functioning is minimised.

It will be readily appreciated that in the light of the present invention a hybrid PKS can be constructed by selecting pieces of DNA encoding respectively a loading module, a variable number of extension modules up to at least six in number, and a chain-releasing thioesterase domain; and concatenating the DNA, using standard procedures, in the order in which it is intended that the gene products operate. NB: The hybrid PKS with (say) 6 modules may be part of an assembly of synthases leading to a product produced by many more than 6 extension modules. It will also readily occur to those skilled in the art that the module-sized DNA fragments may be constituted in more than way, Thus the present invention includes the construction of functional hybrid PKSs exemplified by the construct containing the following activities in a single polypeptide chain:

AT0-ACP0-KS1- [ATR1-DHR1-ERR1-KRR1-ACPR1-KSR2] -AT2-KR2-ACP2  
25 -TE

where the activities shown in square brackets are derived from modules 1 and 2 of the rap PKS, and the rest are

derived from the loading module, extension modules 1 and 2, and the chain-terminating thioesterase of DEBS1. In such constructs, each ketosynthase domain is kept together with the ACP, AT and reductive domains of the module that precedes it in the naturally occurring PKS from which it was derived, rather than with the activities of its own module. Alternative but equally functional arrangements of the module-sized DNA building blocks for construction of hybrid PKS will readily occur to those skilled in the art.

CLAIMS:

99

1. A method for the production of a hybrid PKS gene assembly comprising a loading module and at least one, and  
5 preferably a plurality, of extension modules by assembling together a first nucleic acid portion or portions encoding at least one domain of a first type I PKS with a second nucleic acid portion or portions encoding at least one type I PKS domain which is heterologous to said first PKS.  
10
2. A method according to claim 1 wherein said first portion encodes at least a loading module, while the second portion encodes one or more extension modules.
- 15 3. A hybrid gene assembly as producible by the method of claim 1 or claim 2.
4. A hybrid gene assembly comprising structural gene components operably linked to a promoter which is not  
20 naturally linked thereto and is of a type II PKS.
5. A hybrid gene assembly according to claim 4 wherein the structural gene components are of a type I PKS gene system.
- 25 6. A vector containing a hybrid gene assembly according to claims 3, 4 or 5.
7. A transformant organism containing a hybrid gene

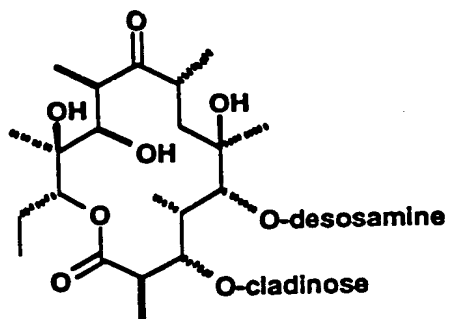
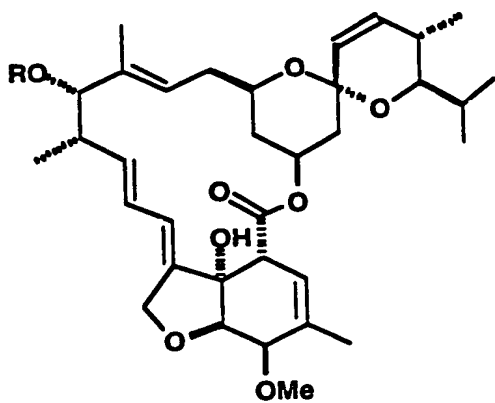
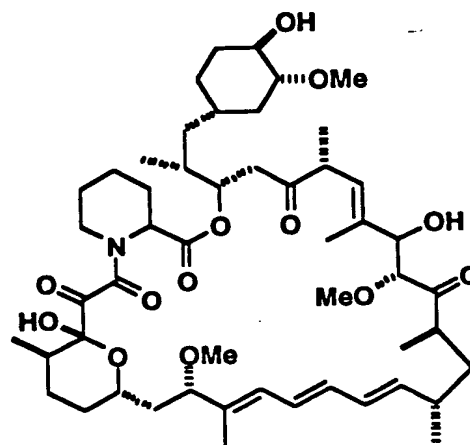
assembly according to claim 3, 4 or 5 and capable of expressing it.

8. A chemical product obtained by culturing the organism of claim 7, said product resulting directly or indirectly from the expression of the hybrid gene assembly.

9. A chemical product according to claim 8 which is a non-natural polyketide.



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**Erythromycin A****Avermectin A1b****Rapamycin****Figure 1.**

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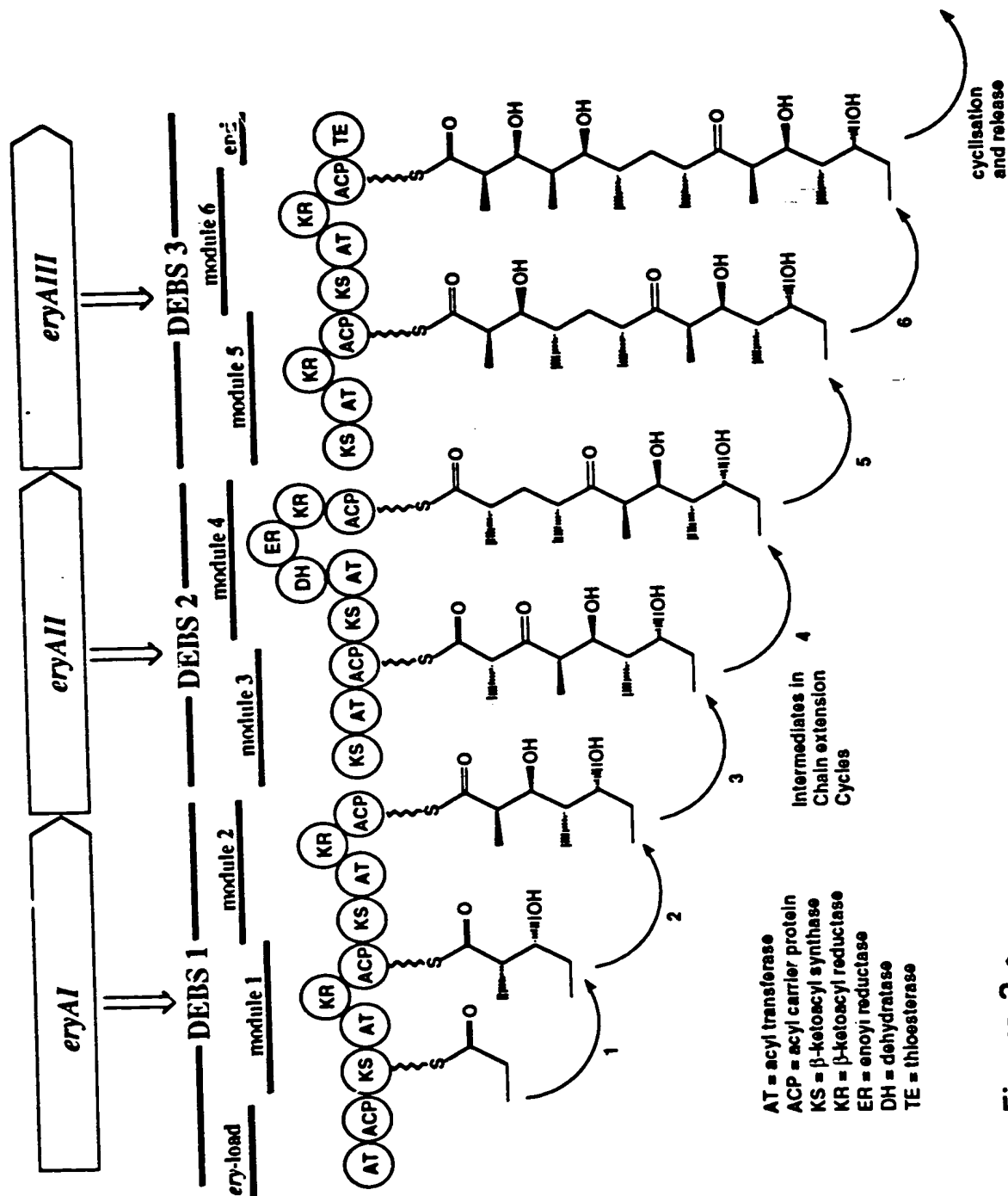
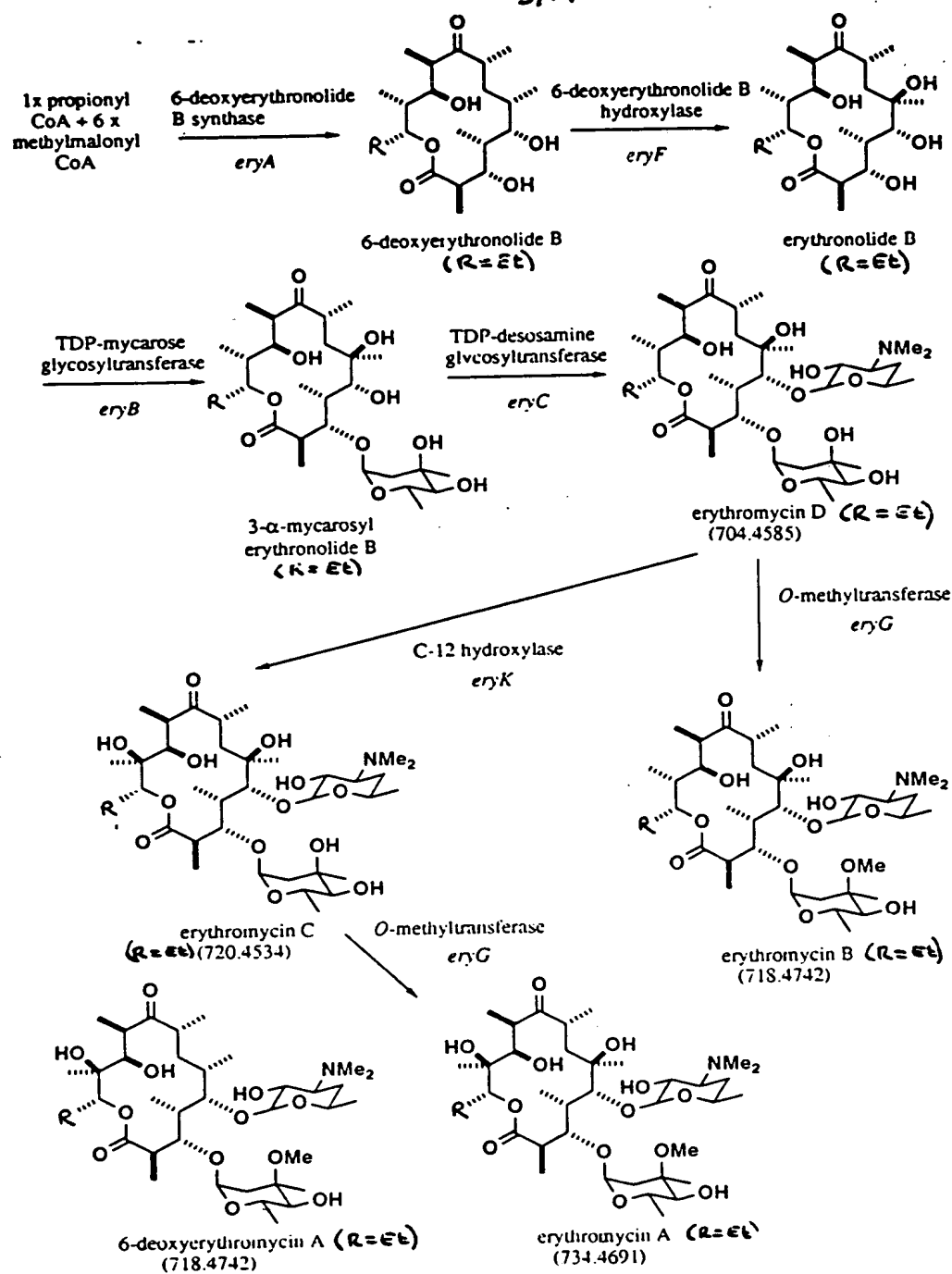


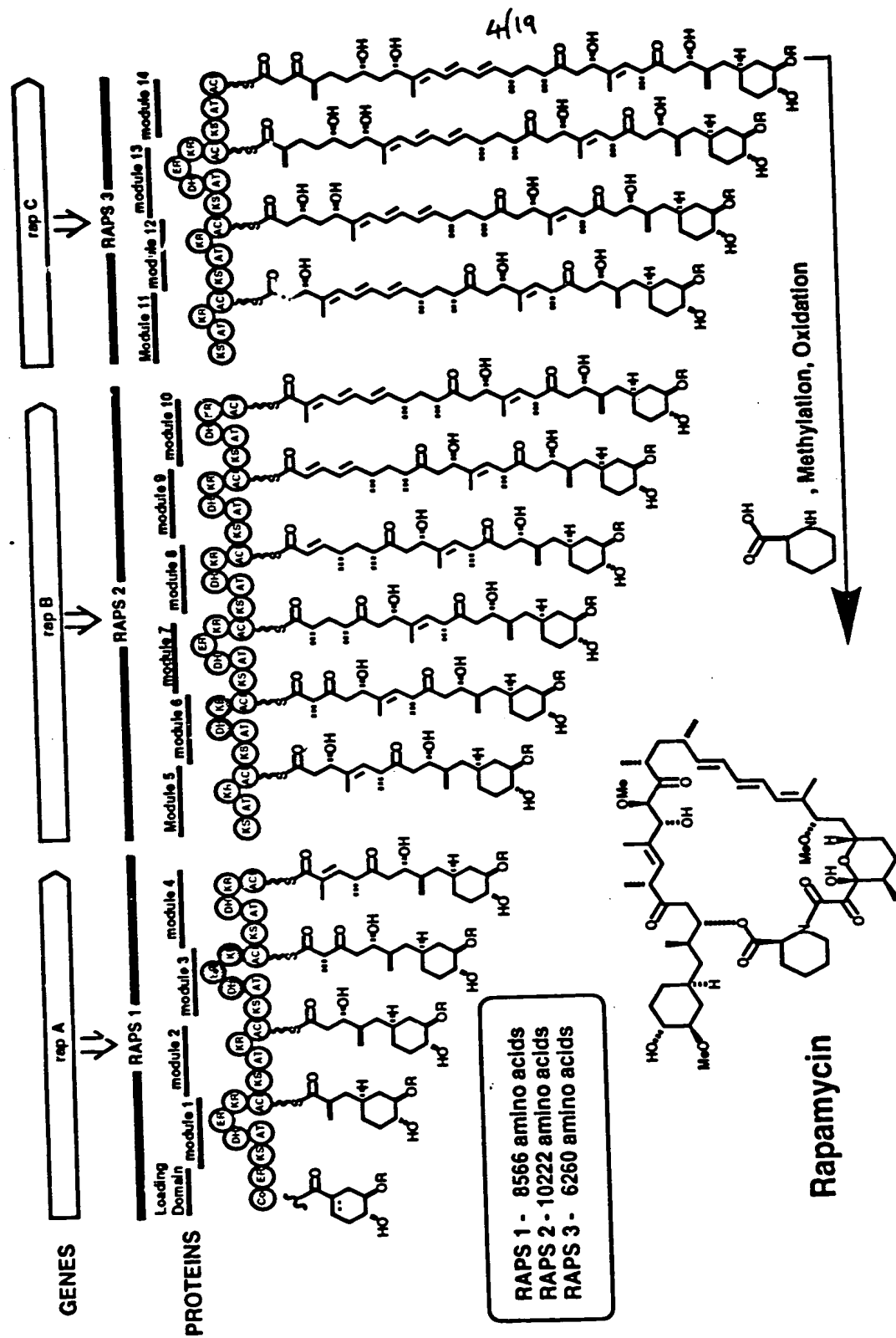
Figure 2 A

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Post-PKS biosynthesis of erythromycins

Fig 2.8



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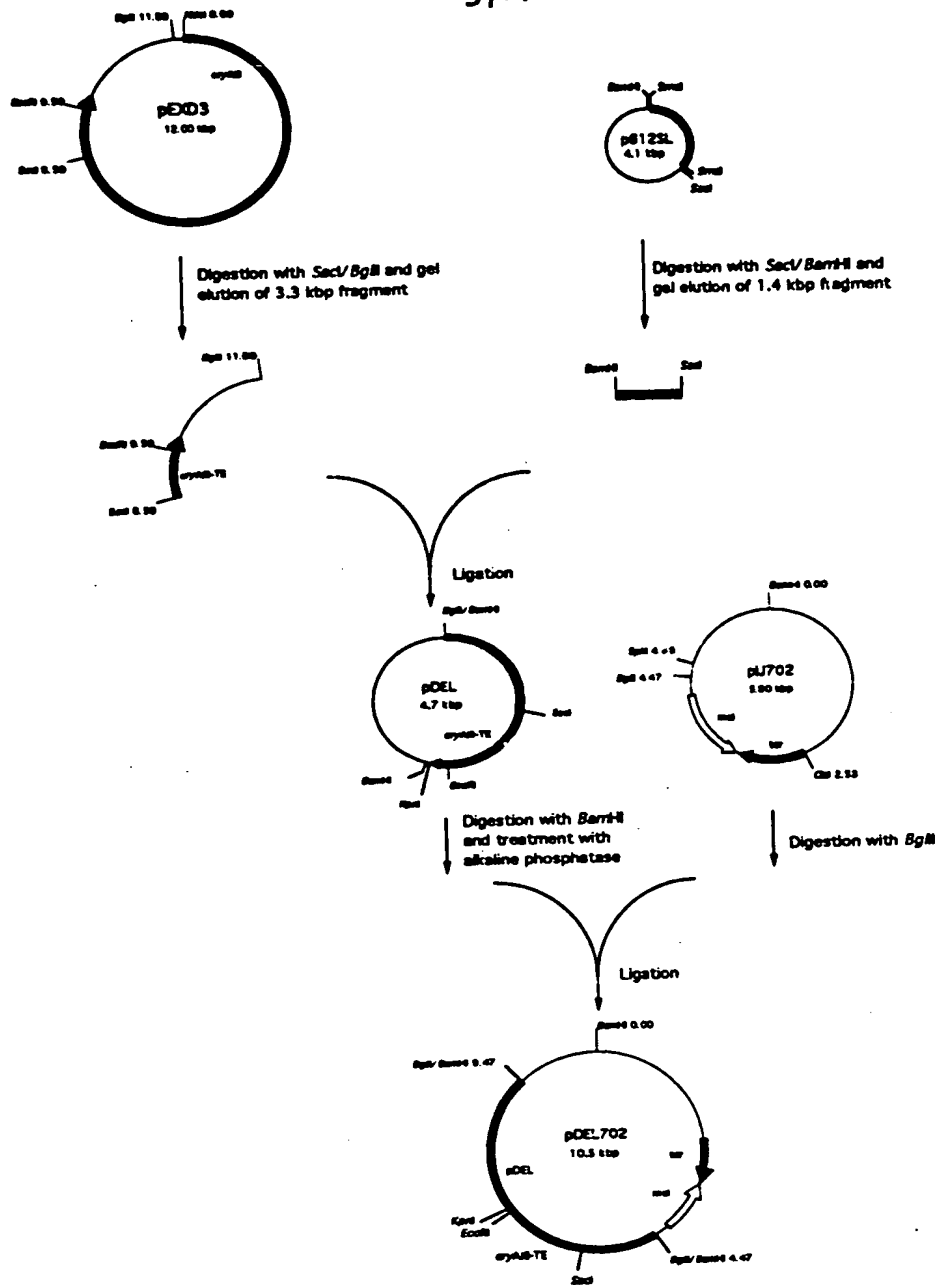
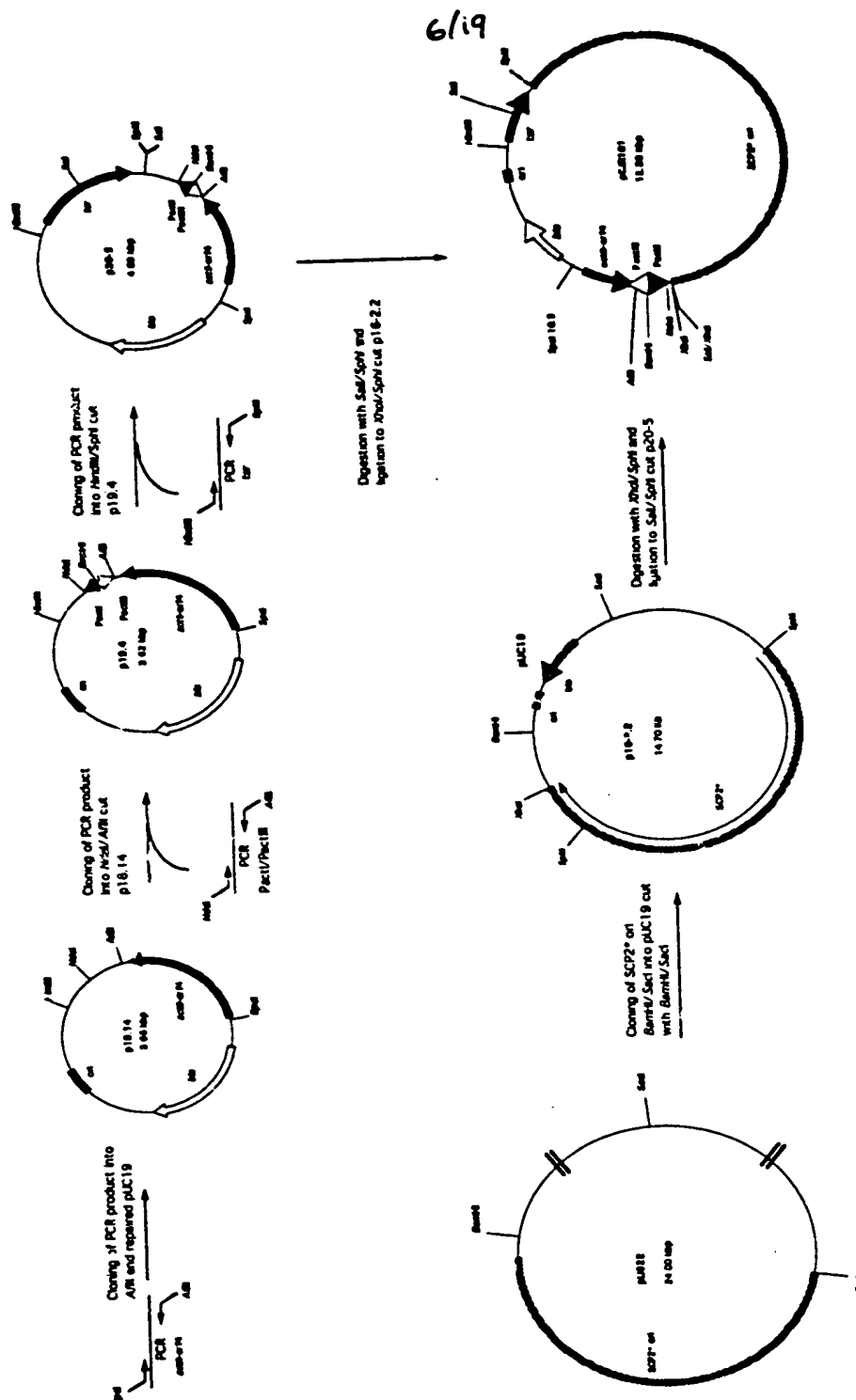


Figure 4



## Figure 5

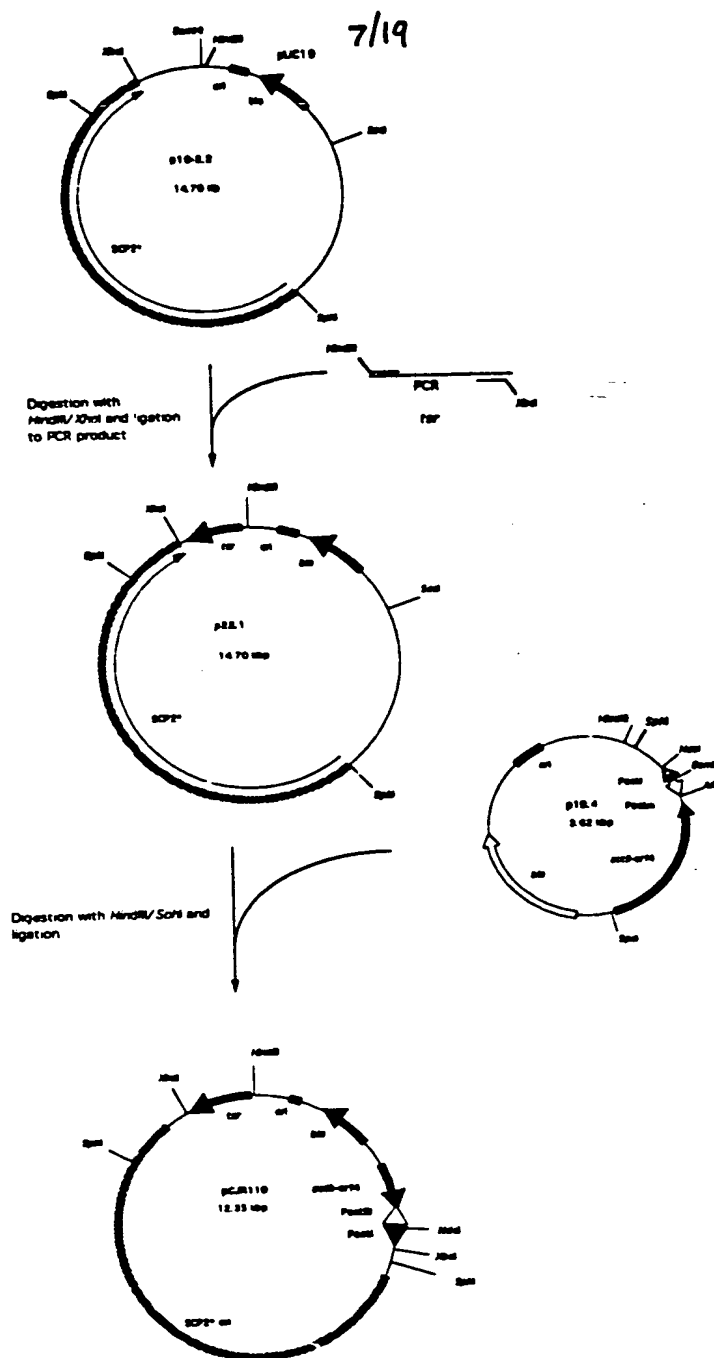


Figure 6.

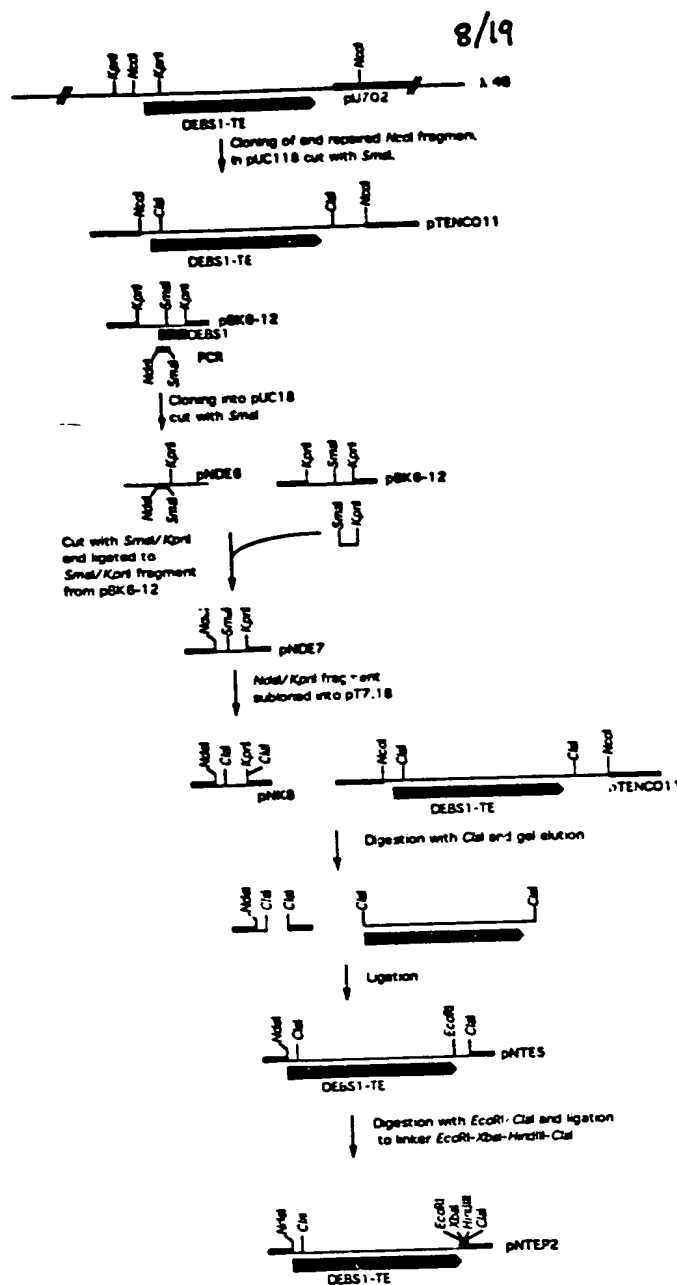
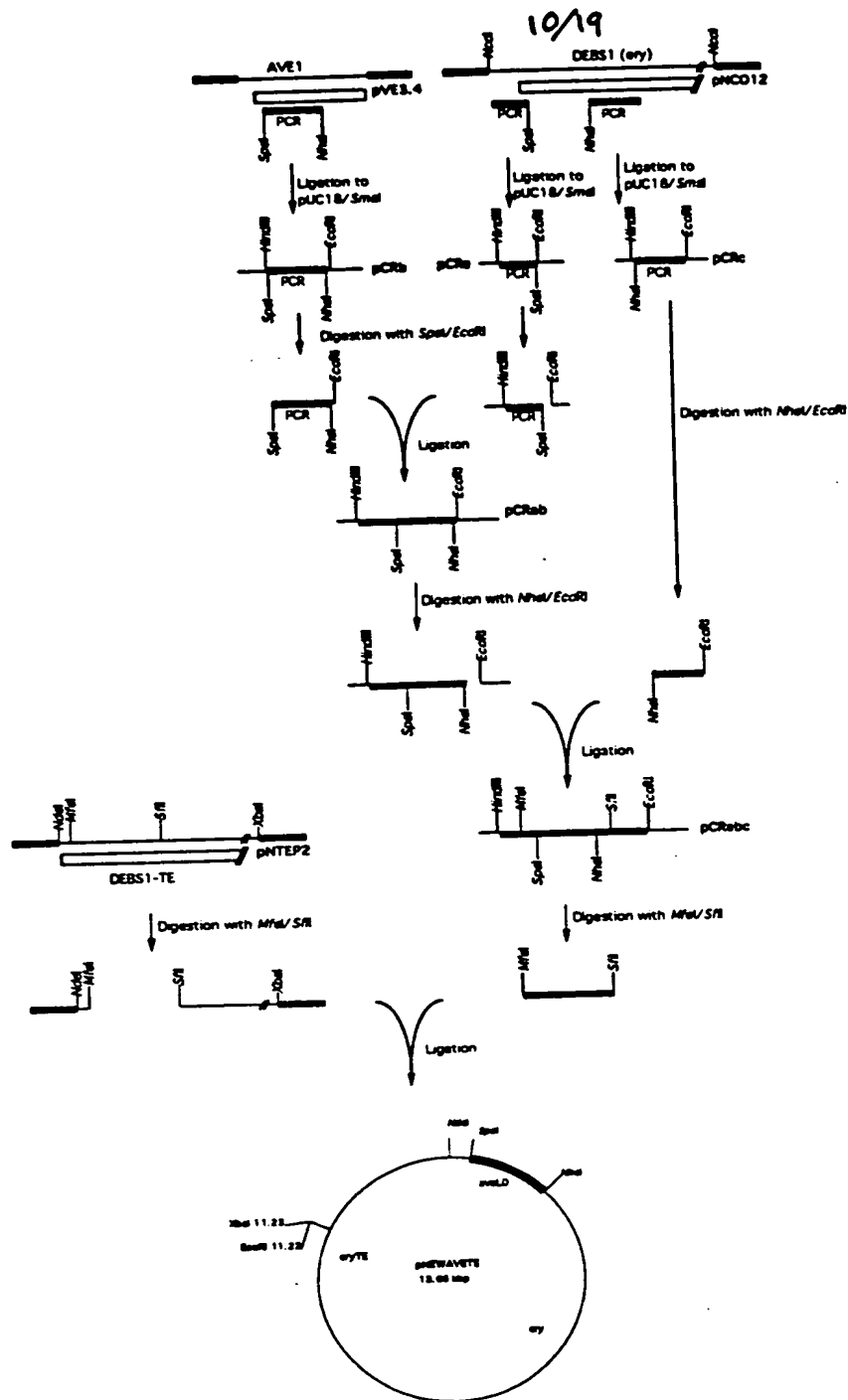


Figure 7.







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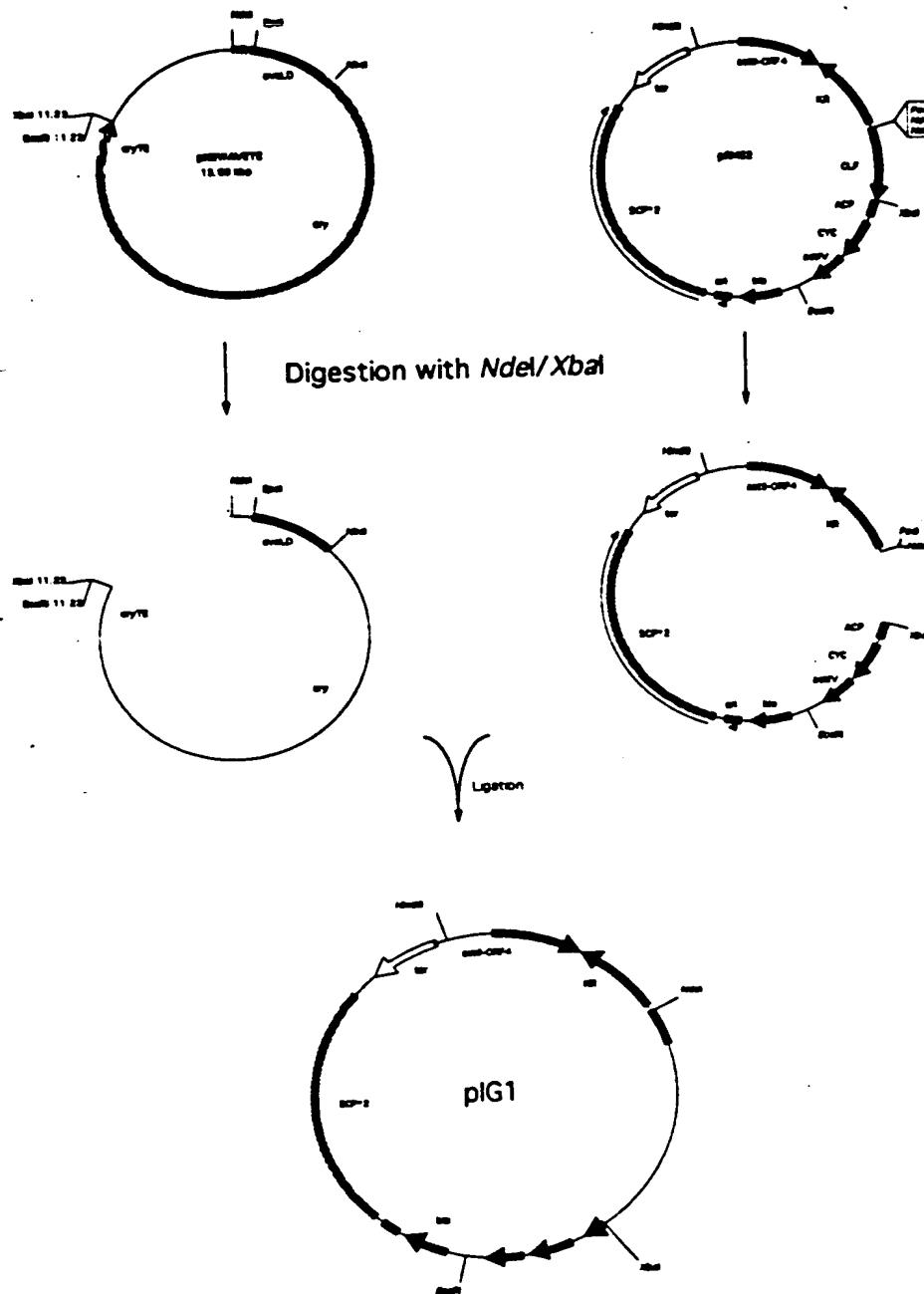


Figure 9b.

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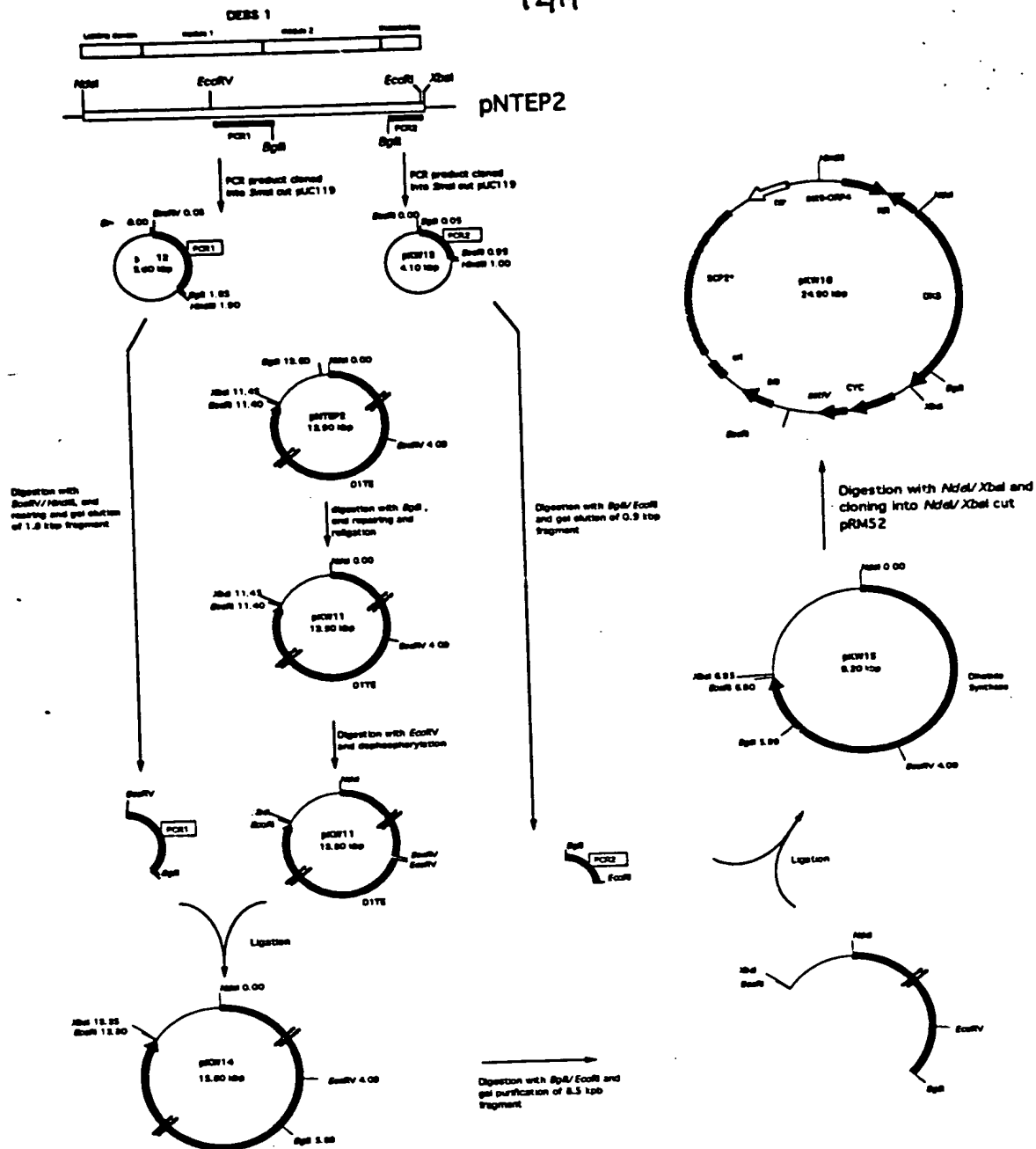


Figure 10.

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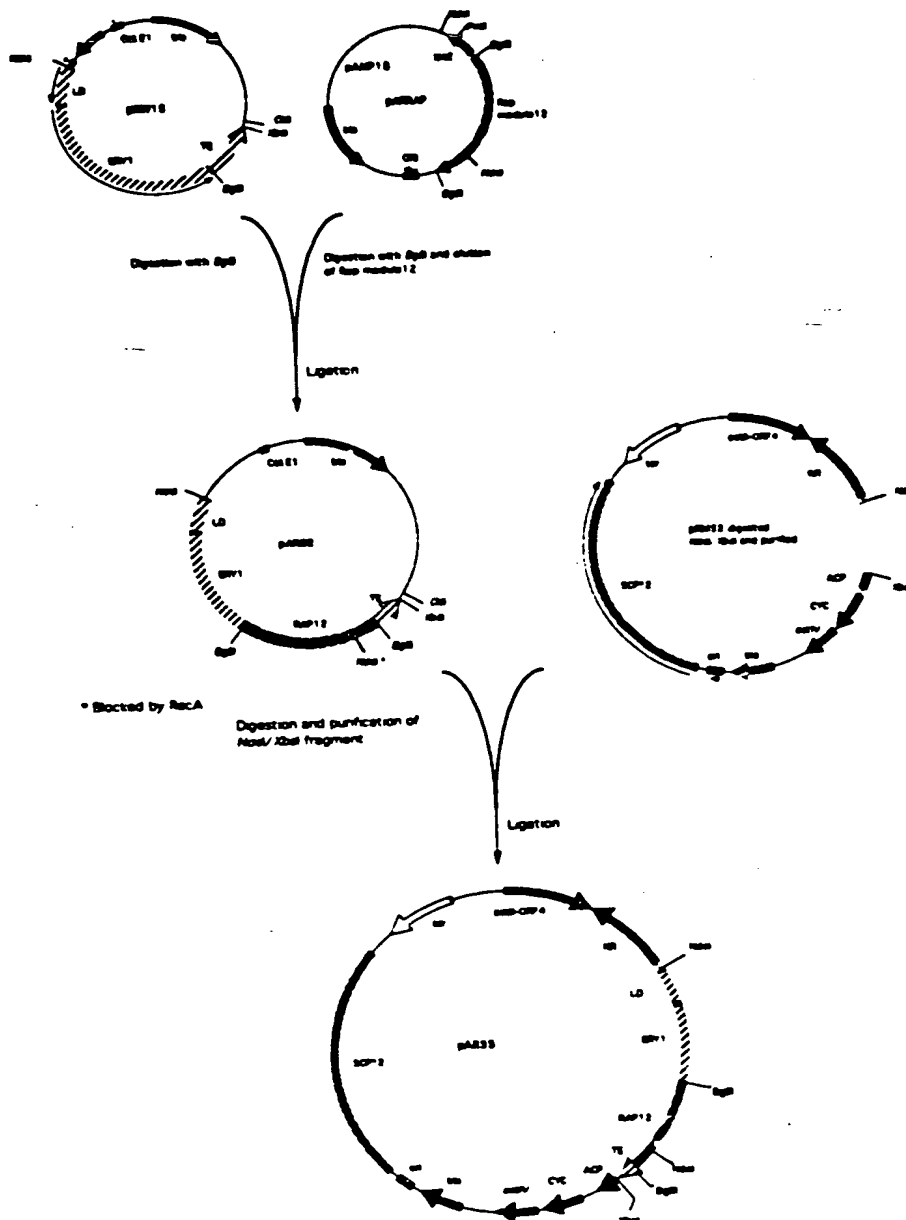


Figure 11.

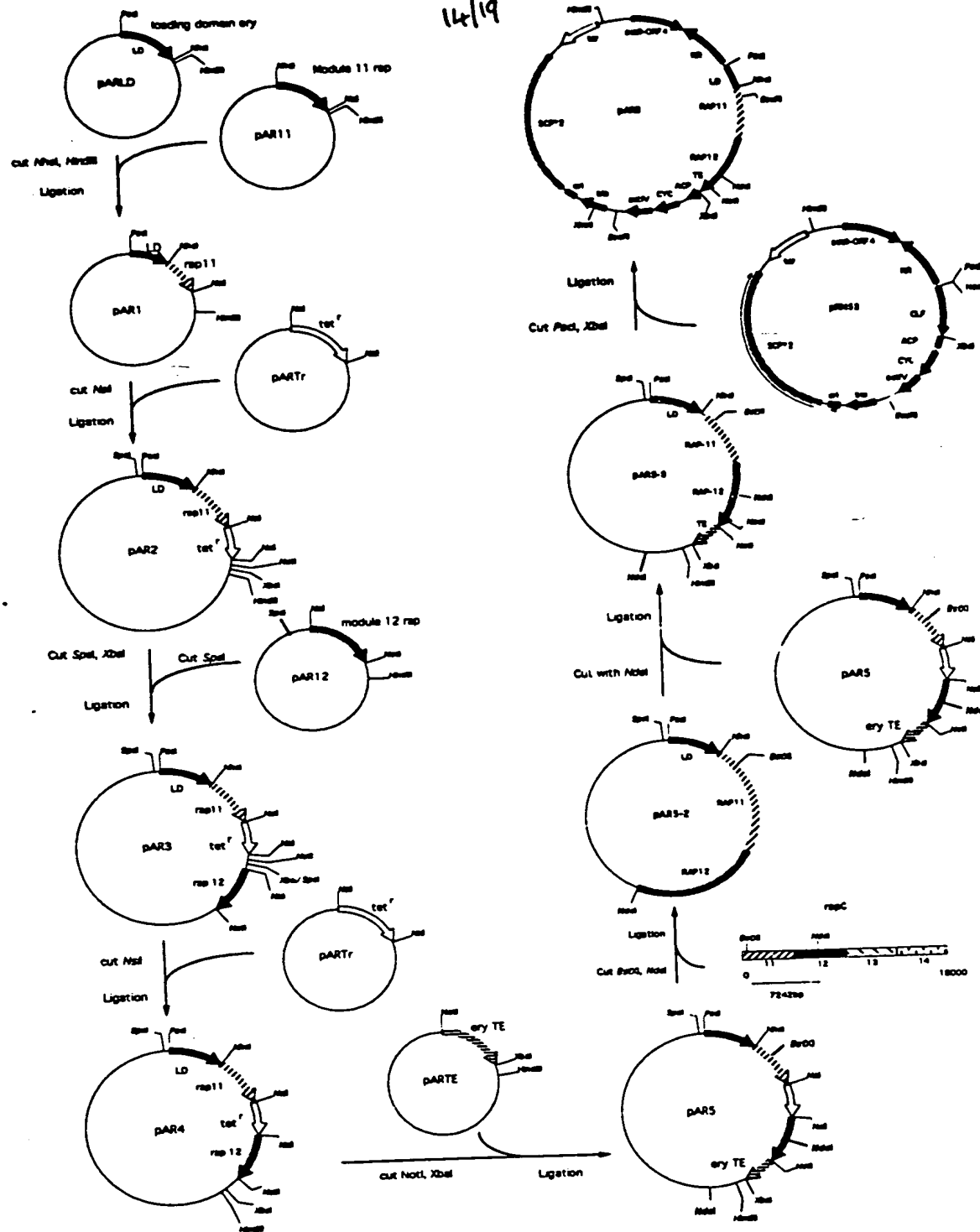


Figure 12.



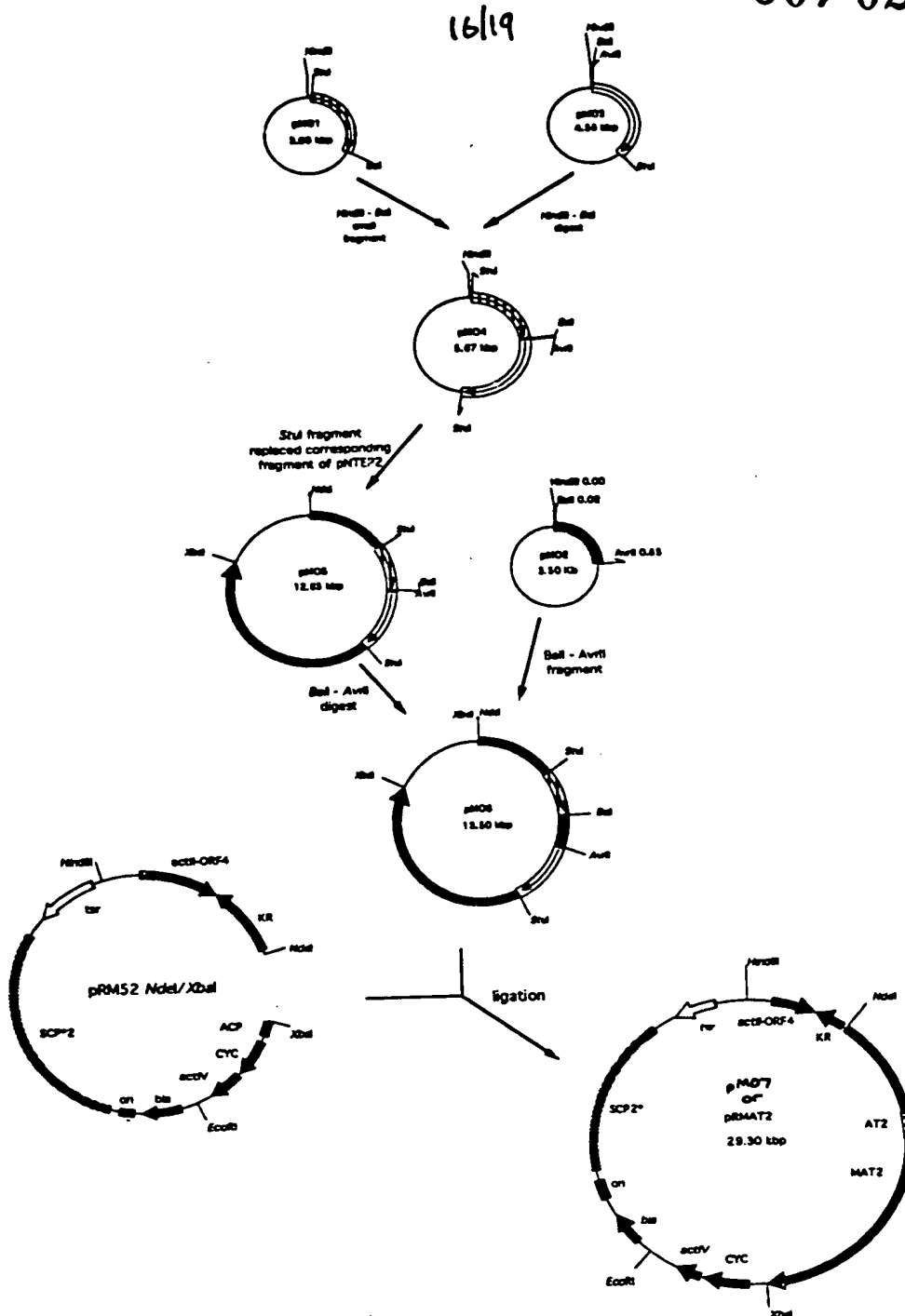


Figure 14.



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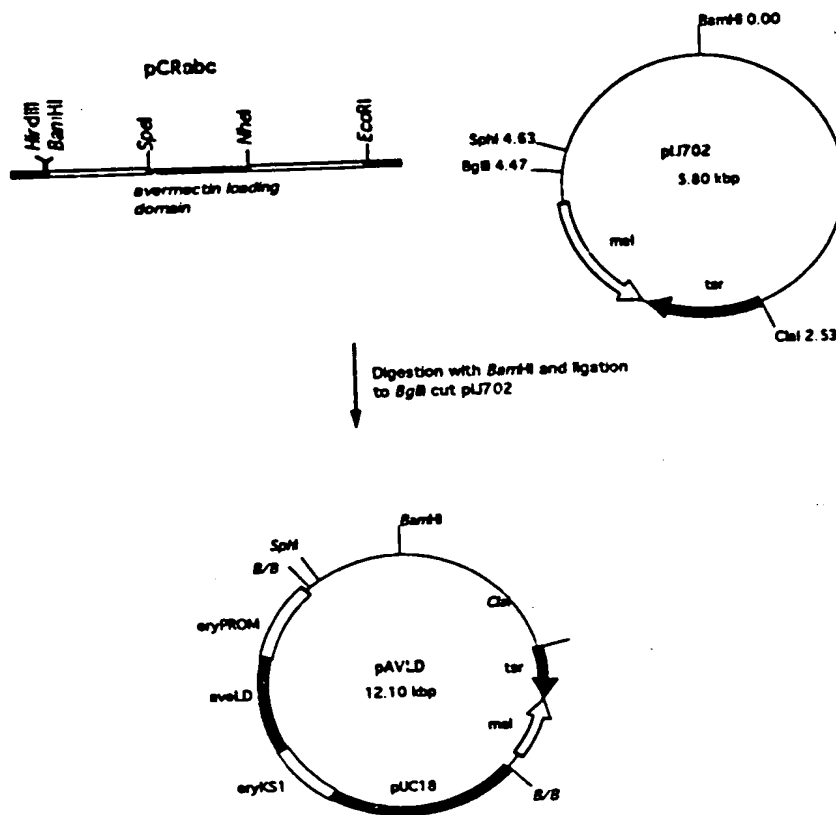


Figure 15.

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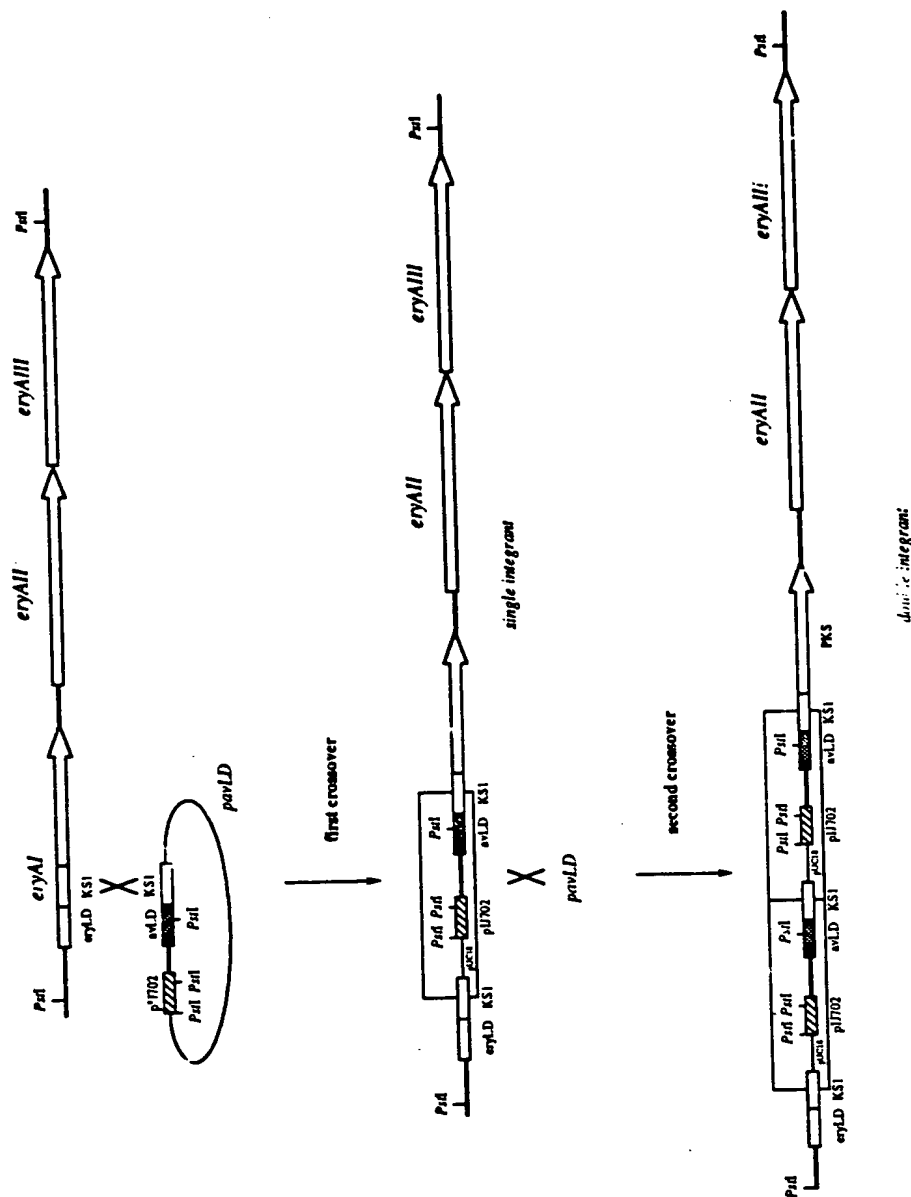


Figure 16.

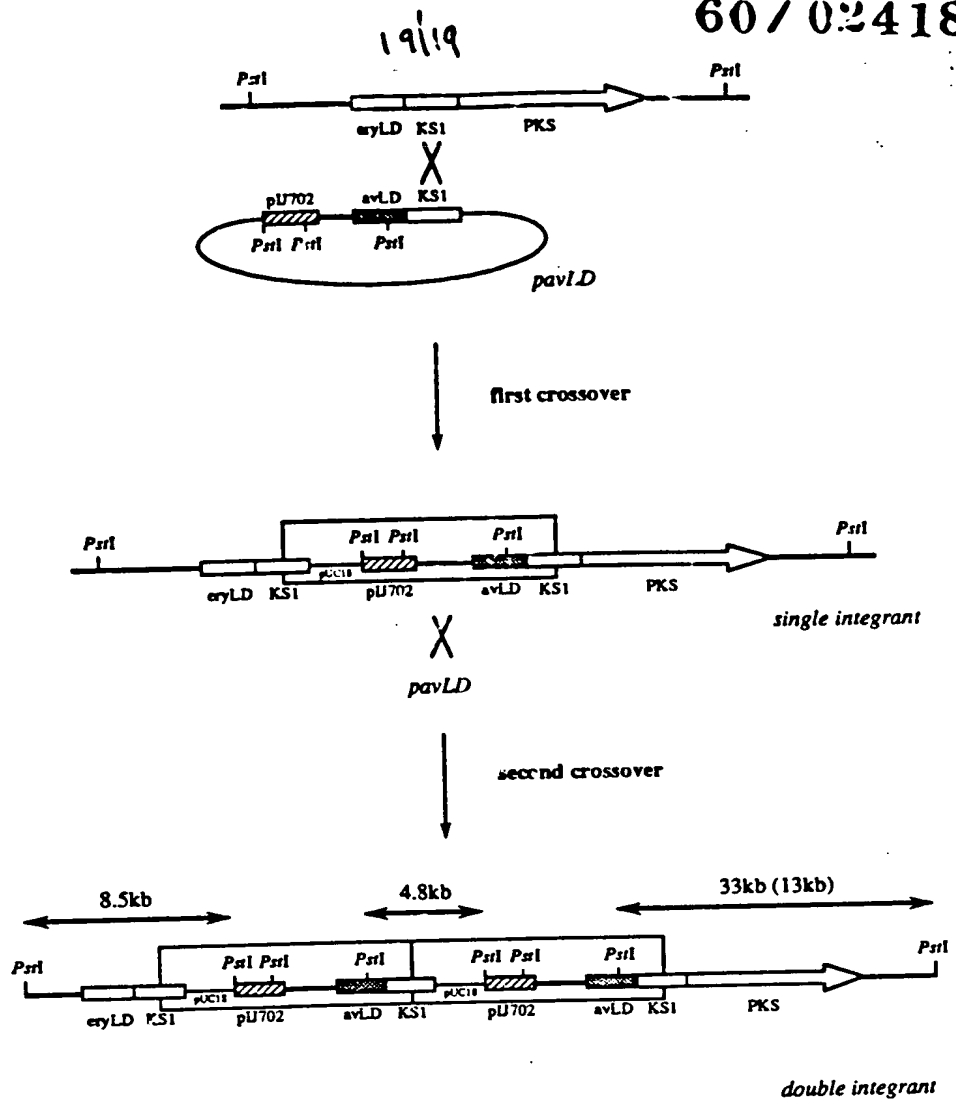


Figure 17.

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